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Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Transgenesis in the Rat and Larger Mammals

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Advances in biotechnology over the last ten years have made it possible for the researcher to alter gene expression in vivo in many diverse ways (1). With the establishment of embryonic stem (ES)[1] cell technology (2), more subtle and precise alterations can now be achieved than were previously possible using microinjection techniques. However, to date germline transmission has only been achieved with mouse ES cells, and microinjection continues to be the method most widely used for other species. While the mouse has a number of advantages, not least the depth of our knowledge of its genetics, other species are being increasingly used for transgenic studies due to their greater suitability for addressing specific questions. We will briefly review the application of transgenic technology to nonmurine species as it stands at present, with particular emphasis on developments appertaining to biomedical research.

Transgenesis by pronuclear injection

A number of significant limitations regarding the application of pronuclear injection to nonmurine animals have been identified (3), not least being the time and cost. Such limitations are due to longer gestation and generation times, reduced litter sizes, and higher maintenance costs. Further consideration must be given to the large numbers of fertilized eggs (and hence donor animals) required for microinjection, the high cost of carrying nontransgenic offspring to term, and the relatively low efficiency of gene integration. Such limitations are particularly severe for the production of bovine transgenics and, as a consequence, more significant departures from the standard procedures used for the mouse have been adopted for this species (4). For example, the use of in vitro embryo production in combination with gene transfer technology has played a large role in the development of transgenic cattle. The development of microinjected embryos through to the morula/blastocyst stage in recipient rabbits or sheep, enables sexing, transgene screening, and cloning to take place before reintroduction into the natural host, providing that such screening methods are robust and reliable.

The major problem regarding pronuclear microinjection is that the exogenous DNA integrates randomly into chromosomal DNA. Position effects, where the transgene is influenced by its site of integration in the host chromosome (5), can have major consequences on the expression of the transgene, including loss of cell specificity, inappropriately high copy number–independent expression and complete silencing of the transgene. This is of greater concern in nonmurine transgenesis where the investment is higher. Position-independent, copy number–related expression can be achieved using sequences such as the locus control regions identified upstream of the β-globin gene cluster and downstream of the CD2 gene (6, 7), the A elements which flank the chicken lysozyme gene (8), and matrix attachment regions (9). Such elements have been shown to function across species barriers, and their incorporation into gene constructs can overcome position effects and improve expression of heterologous genes within specific cell types (5). In many cases, simply including large amounts of flanking sequences may be sufficient to overcome position effects and direct expression to specific tissues. To this end, the development and use of P1 (10), bacterial artificial chromosome (BAC) (11) and yeast artificial chromosome (YAC) vectors (12) for cloning of large segments of DNA, should greatly improve the chances of including important regulatory elements, including those involved in chromatin structure, within the transgene construct.

Embryonic stem cell technology

With the development of ES cell technology in the mouse (2), genetic manipulations can be performed in cell culture using appropriate selection strategies to permit the directed integration of the transgene to a specific region of the chromosome via homologous recombination. With the advent of homologous recombination, the researcher is able to insertionally inactivate, replace, or introduce subtle alterations to the endogenous gene of interest. Once the intended genetic change has been verified, the appropriate ES cells are introduced into blastocysts by microinjection, and, during subsequent gestation, may contribute to the developing embryo. If such a contribution is made, then by definition the resulting animal would be chimeric, being derived in part from the ES cells originating in culture. Assuming that the chimerism extends to the germline, then an appropriate breeding strategy will lead to the recovery of nonchimeric heterozygotes and, if viable, mice which are homozygous for the genetic change.

Most attempts to isolate and culture inner cell mass (ICM) cells from other species are based on the methods used for the

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1. *Abbreviations used in this paper:* DAF, decay accelerating factor; ES, embryonic stem; HAR, hyperacute rejection; ICM, inner cell mass.

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mouse. ES cells are maintained in culture in the presence of mouse-derived differentiation-inhibiting agents, provided either as a media supplement or through cocultivation in the presence of feeder cells. It has been suggested that these mouse-derived agents do not adequately prevent differentiation of stem cells in species other than the mouse, and pluripotent rat ES cells, capable of producing chimeras, were found to grow best on primary rat embryonic fibroblasts as the feeder layer (13). Freshly isolated cells from ICMs have been injected into blastocysts to produce chimeric offspring in both sheep and cattle (14), and their totipotency at this stage is further demonstrated by their ability to produce offspring after transfer into enucleated oocytes (15). Such nuclear transfer techniques are potentially very useful for the production of clonal offspring and would avoid the initial chimeric generation necessitated by the injection of ES cells into blastocysts. Recently, bovine-specific culture methods have shown promise with cells of up to 27 d of age maintaining their ability to direct normal calf development following nuclear transfer (16). However, at the present time the reliable generation of bovine ES cell lines requires the pooling of ICMs from several blastocysts and further efforts are required to enable the long-term culture of clonal bovine ES cells. Although to date chimeric animals have been generated from several species including the pig (17), in no species other than the mouse has germline transmission of an ES cell been successfully demonstrated. This remains a major goal for the future and may well require the use of novel strategies which depart widely from the traditional methods used in the mouse.

Nonmurine species in biomedical research

Selected physiological questions may be more conveniently modelled in the rat or in larger species. Not only can physical size be an advantage for biochemical sampling and physiological analyses, but certain genes may provide useful information when introduced into, for example, the rat genome when parallel experiments in the mouse would be ineffective. Examples include the modulation of blood pressure by the mouse *Ren-2* gene (18) and the modeling of inflammatory disease (19). In both cases, but for different reasons, no phenotype was observed in the respective transgenic mice, highlighting one of the advantages of having alternative species for understanding physiological mechanisms and the etiology of disease. More recently, a number of transgenic experiments have been undertaken to investigate lipoprotein metabolism. The human apolipoprotein A-1 gene was successfully expressed in the rat (20), resulting in increased serum HDL cholesterol concentrations, and attempts to therapeutically lower apo B100, and hence LDL and lipoprotein(a) concentrations, in the rabbit were successful (21) but resulted in complications. Although the targeted expression of the apo B–editing protein in the liver of the transgenic rabbits resulted in reduced LDL and lipoprotein(a) concentrations as intended, many of the animals developed liver dysplasia, suggesting that high level expression of the editing protein had unforeseen and detrimental side effects, possibly via the editing of other important mRNAs. The rabbit has also been used in HIV-1 research, with the development of a line expressing the human CD4 protein on T lymphocytes (22). Susceptibility to HIV infection was demonstrated, and although the rabbits are less sensitive to infection than humans, they may represent an inexpensive alternative to primates for many studies.

Gene transfer in farm animals was initially aimed towards improving production efficiency, carcass quality (23), and disease resistance of livestock. However, it has been suggested that the simple over-expression of hormones such as growth hormone may have unacceptable side effects. Recently some elegant studies of growth using transgenic rats have been performed and are likely to yield valuable information on the biochemistry and physiology of growth (24, 25). A more successful application of transgenesis in farm animals has been the production of biomedically important proteins. The two most popular methods have been to direct expression to hematopoietic cells or to the lactating mammary gland. In the former case, transgenic swine expressing high levels of human hemoglobin were generated using the locus control region from the β-globin gene cluster to overcome positional effects and direct expression to the hematopoietic cells (26). However, due to its natural ability to synthesize and secrete large amounts of protein, the mammary gland has become the primary focus for the expression of heterologous proteins in large mammals. Transgene expression has been successfully directed to the mammary gland using promoter sequences from milk protein genes such as those encoding ovine β-lactoglobulin (BLG), goat β-casein, and murine whey acidic protein. The BLG promoter was used to direct expression of human α_1-antitrypsin in lines of transgenic mice and sheep (27). Interestingly, a wide variation in expression was observed between mouse lines, and from one lactation to another within a single line. In sheep however, similar high levels of heterologous protein were expressed in milk over consecutive lactations and over several generations in a given transgenic line, allowing the viable development of a flock of transgenic sheep. In separate studies high levels of expression of human tissue plasminogen activator were obtained in goat's milk under the control of the goat β-casein promoter (28). The development of suitable purification methods and the use of transgenically produced proteins in clinical trials are well advanced, and, if successful, will have important implications for the production of human proteins in transgenic livestock. Poor expression of the ovine promoter in the mouse may reflect species differences in recognizing heterologous versus homologous promoters and raises questions concerning the predictive value of mouse models. At best therefore the generation of transgenic mice may, in certain cases, only be a guide to the potential success of a transgene construct in another species.

Gene transfer could equally be used to enhance the quality and suitability of milk derived from domesticated animals as a food for human consumption. Human milk is devoid of β-lactoglobulin, which is responsible for most of the allergies to cows' milk, and has a relatively high content of lactoferrin, which is important in iron transport and combating bacterial infections. One could envisage in the future the reduction of saturated fat content in cows' milk and the knock-out of unwanted proteins or their replacement with other more useful components. Through the manipulation of milk constituents it should be possible to more closely emulate the desirable components of human milk. The alteration of milk composition would appear to be a practical possibility given that milk micelles are remarkably tolerant to changes in composition, as demonstrated by the knock-out of the mouse β-casein gene (29). Ethical concerns regarding the generation of transgenic animals, which have been engineered specifically for pharmaceutical, medical, or nutritional reasons, lie outside the scope

of this overview, however it must be clearly ascertained that expression of a transgene does not compromise the animal.

Xenograft organs for transplantation surgery

The shortage of human organs for transplantation has raised interest in the possibility of xenotransplantation, i.e. the use of animal organs (30). However, the major barrier to successful xenogeneic organ transplantation is the phenomenon of complement-mediated hyperacute rejection (HAR), brought about by high levels of circulating natural antibodies that recognize carbohydrate determinants on the surface of xenogeneic cells. After transplantation of the donor organ, a massive inflammatory response ensues through activation of the classical complement cascade. This leads to activation and destruction of the vascular endothelial cells and, ultimately, the donor organ. The membrane-associated complement inhibitors, endogenous to the donor organ, are species restricted and thus confer only limited resistance. The complement cascade is regulated at specific points by proteins such as decay accelerating factor (DAF), membrane cofactor protein, and CD59. These regulators of complement activation are species specific. The initial strategy used to address HAR in porcine-to-primate xenotransplantation was to produce transgenic pigs expressing high levels of the human terminal complement inhibitor, hCD59. This was shown to protect the xenogeneic cells from human complement-mediated lysis in vitro (31). More recently, organ transplantation has been achieved using donor pigs which expressed human DAF on their endothelium (32), or both DAF and CD59 on erythrocytes, such that the proteins translocated to the cell membranes of endothelial cells (33). After transplantation, the pig hearts survived in recipient baboons for prolonged periods without rejection (33). Clearly, such genetic manipulations are bringing xenotransplantation ever closer to reality. If the isolation of suitable ES cells and application of homologous recombination becomes a reality in the pig, it may be possible to knockout the antigenic determinants to which antispecies antibodies bind, as a further strategy for eliminating HAR.

Summary

The use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given construct may react very differently from one species to another. The application of transgenesis in the pig should produce major advances in the fields of transfusion and transplantation technology, while alterations in the composition of milk in a range of domesticated animals will have major effects on the production of pharmacologically important proteins and could eventually lead to the development of human milk substitutes. Despite the lack of germline transmission to date, major efforts continue to be directed towards the generation and use of ES cells from nonmurine species, using both traditional and new technologies, and the availability of such cells is likely to accelerate both the use of such species and the precision with which genetic changes can be introduced.

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of this overview, however it must be clearly ascertained that expression of a transgene does not compromise the animal.

Xenograft organs for transplantation surgery

The shortage of human organs for transplantation has raised interest in the possibility of xenotransplantation, i.e. the use of animal organs (30). However, the major barrier to successful xenogeneic organ transplantation is the phenomenon of complement-mediated hyperacute rejection (HAR), brought about by high levels of circulating natural antibodies that recognize carbohydrate determinants on the surface of xenogeneic cells. After transplantation of the donor organ, a massive inflammatory response ensues through activation of the classical complement cascade. This leads to activation and destruction of the vascular endothelial cells and, ultimately, the donor organ. The membrane-associated complement inhibitors, endogenous to the donor organ, are species restricted and thus confer only limited resistance. The complement cascade is regulated at specific points by proteins such as decay accelerating factor (DAF), membrane cofactor protein, and CD59. These regulators of complement activation are species specific. The initial strategy used to address HAR in porcine-to-primate xenotransplantation was to produce transgenic pigs expressing high levels of the human terminal complement inhibitor, hCD59. This was shown to protect the xenogeneic cells from human complement-mediated lysis in vitro (31). More recently, organ transplantation has been achieved using donor pigs which expressed human DAF on their endothelium (32), or both DAF and CD59 on erythrocytes, such that the proteins translocated to the cell membranes of endothelial cells (33). After transplantation, the pig hearts survived in recipient baboons for prolonged periods without rejection (33). Clearly, such genetic manipulations are bringing xenotransplantation ever closer to reality. If the isolation of suitable ES cells and application of homologous recombination becomes a reality in the pig, it may be possible to knockout the antigenic determinants to which antispecies antibodies bind, as a further strategy for eliminating HAR.

Summary

The use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given construct may react very differently from one species to another. The application of transgenesis in the pig should produce major advances in the fields of transfusion and transplantation technology, while alterations in the composition of milk in a range of domesticated animals will have major effects on the production of pharmacologically important proteins and could eventually lead to the development of human milk substitutes. Despite the lack of germline transmission to date, major efforts continue to be directed towards the generation and use of ES cells from nonmurine species, using both traditional and new technologies, and the availability of such cells is likely to accelerate both the use of such species and the precision with which genetic changes can be introduced.

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TRANSGENIC LIVESTOCK: PROGRESS AND PROSPECTS FOR THE FUTURE

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ABSTRACT

The notion of directly introducing new genes or otherwise directly manipulating the genotype of an animal is conceptually straightforward and appealing because of the speed and precision with which phenotypic changes could be made. Thus, it is of little wonder that the imagination of many an animal scientist has been captivated by the success others have achieved by introducing foreign genes into mice. The private sector has embraced transgenic livestock technology resulting in the formation of two new industries. However, before transgenic farm animals become a common component of the livestock production industry, a number of formidable hurdles must be overcome. In this brief communication, the technical challenges are enumerated and possible solutions are discussed.

Key words: transgenic livestock, gene transfer, microinjection

INTRODUCTION

The definition of transgenic animals is evolving. For the purpose of this paper a transgenic animal is one containing recombinant DNA molecules in its genome that were introduced by intentional human intervention. In this review I will focus on animals in which transgenes were introduced into preimplantation embryos by pronuclear microinjection, with the intended consequence of producing germline transgenics as opposed to somatic cell transgenics. Though there are other means of introducing genes into preimplantation embryos (20,29), pronuclear microinjection, basically as originally described by Jon Gordon (25), and as modified for livestock in our laboratory (65), is still the predominant method employed.

Acknowledgments

Many of the concepts, conclusions and visions of the future included in this manuscript have evolved over the years from discussions at our Friday afternoon lab meeting. Vern Pursel and Caird Rexroad, Jr., who pioneered transgenic livestock technology, provided the leadership. In recent years Ken Bondioli, David Kerr, Paul Hyman and Uli Tillmann have provided valuable new insights and new approaches that have and will advance the field.

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WHY MAKE TRANSGENIC ANIMALS?

A Medline search reveals that over 6,000 scientific articles have been published in which transgenic animals (mostly mice) were used to answer basic research questions. By contrast 289 papers dealt with transgenic livestock, of which 24% were reviews. The limited publication record for transgenic livestock species reflects the high costs and technical difficulties associated with producing transgenic livestock more than lack of applicability of this technology to farm animals. A number of well defined goals have been enumerated in the numerous review articles written by animal scientists. Not surprisingly, many of the proposed applications closely parallel the long term objectives of animal agriculture.

In theory, transgenic technology provides a mechanism by which economically important traits can be attained more rapidly than by selective breeding without concern of propagating associated, possibly undesirable, genetic characteristics. If genetic precision and speed of improvement were the only advantages of transgenic technology, use of that methodology might be difficult to justify. That is because current cost of producing transgenic animals are high and understanding of the appropriate genetic manipulations required to influence economically important traits is limited. However, transgenic technology offers much more. Genes can be transferred across species boundaries and can be modified to function very differently than they do in their native form (gene products, tissue specificity, and timing of expression can be altered). The ability to redirect expression of genes to another organ has spawned the transgenic bioreactor industry. For the most part, transgenic bioreactors are farm animals designed to produce new proteins in their milk or other body fluids. It is envisioned that this approach will have application in both food production and the biomedical arena. Modifying the composition of milk through genetic engineering is the topic of Dr. Bremel's paper in these proceedings and will not be dealt with here.

TRANSGENIC LIVESTOCK PROJECTS

For the sake of brevity, only a very brief summary of the 37 gene constructs that have been tested in livestock will be reported here. The reader is referred to two excellent reviews that list those constructs and their consequences (16,53).

The Transgene.

The power of transgenic technology is derived from the introduction of genetic information with new functionality. The strategy for building a transgene (fusion gene) involves selecting a genetic regulatory element (often called promoters, but usually containing both an enhancer element and a promoter) that will determine the tissue in which the gene is to be expressed and the time and magnitude of expression. In some cases, the regulatory element can act as a switch, allowing the transgene to be turned on and off at will. The second part of the gene construct consists of DNA sequence encoding the desired protein (often referred to

as the structural component of the transgene). For example, in the first transgenic livestock experiment (28) we wanted to increase the levels of circulating growth hormone in a controlled manner. The gene construct used to accomplish this consisted of the regulatory element of a metallothionein (MT) gene fused to the coding sequence for growth hormone (GH). Metallothionein is an inducible liver enzyme, and its gene is usually quiescent (turned off) until a threshold level of circulating zinc or cadmium triggers transcription. Therefore, it was expected that the MT-GH fusion gene would be silent until the animals were fed zinc. In those experiments GH expression could be inducted but, in most cases, the transgene could not be turned off completely. New more complex inducible approaches are now being tested (23,26). These new systems rely on tetracycline or its analogs to activate or repress transgene expression. It is too early to know if these strategies, in their current form, will be more tightly regulated then the MT system. However, if they are not, the general paradigm on which the new systems are based will probably lead to improved inducible systems

Published Transgenic Projects.

The vast majority of original research reports have focused on growth enhancement. Growth hormone (GH) was the structural gene employed in 13 of those publications and the gene for growth hormone releasing factor in four. Other structural genes tested include IGF-1, cSKI and an estrogen receptor. The regulatory elements derived from MT genes, from various species, were most frequently used appearing in nine of the growth-related fusion genes. Long terminal repeats (LTR) from two retroviruses, MLV and RSV, and sequence from CMV, a DNA virus, served as regulatory components of transgenes, as have the promoters from albumin, prolactin, skeletal actin, transferrin and phosphoenolpyruvate carboxykinase (PEPCK) genes. All but two of 21 growth constructs were tested in pigs and the most striking phenotypes resulted from the use of MT-GH fusion genes (53).

Seven transgenes designed to enhance disease resistance and to produce immunologically-related molecules have been introduced into pigs and sheep (5,13,41,67). Though desirable expression patterns have been reported in several of the projects, none of the studies has progressed to the point of demonstrating a beneficial effect of transgene products.

Very recently it has been reported that transgenic sheep with enhanced wool production characteristics have been produced (9). The results are quite promising; if no unforeseen anomalies occur, transgenically produced wool maybe the first marketed livestock product.

Biomedical Transgenic Projects.

Other proposed transgenic farm animal applications are decidedly non-agricultural in nature. One of the first transgenic animal companies demonstrated the feasibility of producing new animal products by manufacturing human

hemoglobin in pigs, to serve as a principal component of a human blood substitute (59). Human antibodies have also been produced in transgenic mice (62). Another area where transgenic animals, especially pigs, will have a significant impact on society will be in the development of human genetic disease models. To date, genetic disease models have been generated in mice for atherosclerosis (6), sickle cell anemia (18), Alzheimer's disease (21), autoimmune diseases (44), lymphopoiesis (33), dermititis (55), and prostate cancer (61). These models for the most part require "knocking out" the function of a gene or replacing an existing gene with a mutant form. Many of these models will have to be replicated in farm animals to be useful. Unfortunately, the stem cell technology required to generate most of the disease models is still in development for livestock (51).

Finally, a new use not reported in the above mentioned reviews deserves note. The objective of this new endeavor is to genetically engineer animals, primarily pigs, so that their organs can be used as xenografts for humans. Preliminary studies to test the concept have been performed in mice (40,42) and transgenic pigs have now been produced (19,54). Though several strategies are being explored, the general approach has been to block activation of complement, which is normally part of the acute transplantation rejection response. These organs are intended for temporary use, until an appropriate human organ becomes available. However, as the technology develops, a driving force will be the design of transgenic organs for extended use or permanent transplantation.

CHARACTERISTICS OF TRANSGENIC ANIMALS

Transgenic livestock projects are costly, primarily because the process is inefficient. Production costs range from $25,000 for a single founder pig to over $500,000 for a single functional founder calf (64). The calculation for cattle was based on obtaining zygotes by superovulation of embryo donors, the normal practice for all mammalian species. However, the costs are reduced by as much as a third if oocytes derived from ovaries collected at slaughter are the starting material. The remainder of this review will be devoted to characterizing the transgenic animal model, to identify points in the process that reduce efficiency, and finally discussing possible approaches that have been proposed to overcome major hurdles to progress.

Transgene Integration.

Even though several hundred copies of a transgene are microinjected, any transgene that becomes incorporated into the genome generally does so at a single location. Exceptions are rare (58). Thus, transgenic founder animals are hemizygous for transgenes. It is also common for a transgene locus to contain multiple copies of the transgenes arranged in a head-to-tail array. These two characteristics of transgene loci should provide clues to the mechanism by which transgenes integrate. So far, few researchers have formulated compelling hypotheses to explain the event (2,47) and the hypotheses that have been proposed remain untested

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and rats, Table 1).

Table 1. Examples
several laboratories.

Species	Injected & transferred embryos (No.)
Mice	12,314
Rabbits	1,907
Rat	1,403
Cattle[c]	1,018
Pigs	19,397
Sheep	5,424

[a] Number of experiments
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Without knowledge of the molecular mechanism it is going to be extremely difficult to devise approaches to make transgene integration more efficient.

Transgene integration efficiency is low and ranges from about 1% in farm animals (cattle, sheep and pigs) to about 3% in laboratory animals (mice, rabbits and rats, Table 1).

Table 1. Examples of embryo survival and transgene integration efficiencies from several laboratories.

Species	Injected & transferred embryos (No.)	Studies[a] (No.)	Transgenic animals produced: Offspring[b] (No.)	Transgenic animals produced: Per Offspring (%)	Transgenic animals produced: Per embryo injected & transferred (%)	Refs.
Mice	12,314	18	1847	17.3	2.6	(63)
Rabbits	1,907	1	218	12.8	1.5	(28)
Rat	1,403	5	353	17.6	4.4	(45)
Cattle[c]	1,018	7	193	3.6	0.7	(30)
Pigs	19,397	20	1920	9.2	0.9	(53)
Sheep	5,424	10	556	8.3	0.9	(53)

[a] Number of experiments, which in most cases was equivalent to number of different gene constructs tested.

[b] The value for cattle includes both fetuses and live born calves.

[c] Eleven thousand two hundred and six eggs were microinjected and cultured. One thousand and eighteen developed to morula or blastocysts and were transferred into recipient cows.

Transgene Expression

Even after the one in 33 to one in 150 injected and transferred eggs results in a transgenic animal the efficiency of the process is further diminished by failure of the transgene to be transcribed. Transgenes are expressed (transcribed) in only about half of transgenic lines, though some specific transgenes are expressed in a higher proportions (15 ?7). If a founder expresses its transgene, so do its transgenic offspring. It is not clear why some transgenes are expressed in all lines and others in only half the lines. Transgenes are sometimes activated in unintended tissues (ectopic expression), and timing of expression can be shifted relative to development. Our lack of understanding of essential genetic control elements makes it difficult to design transgenes with predictable behavior. The abbarent expression patterns (no expression or wrong expression) seen in some linese of transgenic animals has been attributed to the so-called "position effect." If a transgene lands near highly active genes, the transgene's behavior maybe influenced by endogenous genes. Other transgenes may locate in transcriptionally inactive (heterochromatin) regions. The transgene may function normally or be completely silenced by the

heterochromatin. It is likely that both of these factors (position effect and unidentified control elements) contribute to lack of transgene expression in some lines and variable expression in other lines. Some of these problems will be obviated by use of "boundary" DNA sequences that block the influence of surrounding genes (34,43). Refining transgenic technology for farm animals will remain a challenging task in part because experimentation will often have to be conducted in the species of interest. That is because transgene expression and the physiological consequences of transgene products in livestock are not always accurately predicted in transgenic mouse studies (28,48).

Transgene transmission.

Because founder animals are usually single integrant hemizygous for the transgene, one would expect 50% of their offspring to inherit a copy of the transgene locus. This is true for about 70% of transgenic founder mice (49). The remaining founders either do not transmit transgenes to their offspring or transmit transgenes at a low frequency (52,53). It is commonly thought that the non-Mendelian inheritance is the result of transgene mocasicism in germ cells. This could be caused by late integration of transgenes during embryonic development (60). It has been proposed that non-Mendelian inheritance patterns can also be caused by diminished fertilizing ability of transgene bearing sperm (17). The latter explanation may be a special case, because the thymidine kinase gene used in that study was inadvertently expressed in testes.

POTENTIAL SOLUTIONS FOR IMPROVING EFFICIENCY

Testing Transgenes.

Because the "rules" for transgene design are still vague, it is important to have a reliable system for testing gene constructs. The most cost effective method of characterizing the performance of a transgene is cell culture transfection studies. Unfortunately, such studies have a low predictive value (50). The next most cost effective method for testing gene constructs is production of transgenic mice, which as mentioned above do not faithfully predict a transgene's performance in livestock species. Nevertheless, a reasonable amount of useful information about transgene function can be derived from transgenic mouse studies. Currently, the only approach that yields truly informative data is testing transgenes in the livestock species of interest. This is obviously an unsatisfactory, time consuming, expensive testing option. One alternative approach that we are exploring is based on the fact that transgenes will function after being "shot" into somatic tissue. We have been focusing our efforts on the mammary gland, but almost any target organ should be amenable to this approach. We have recently demonstrated that both RNA and protein can be detected following introduction of transgenes into sheep mammary tissue, *in situ* (22,37). Once we confirm that "gene-gunned" transgenes function as they do in transgenic animals, this approach should dramatically reduce the costs and time of evaluating gene constructs.

Improving Integration Frequency

From Table 1 it is clear that integration rates are lower for livestock species than for laboratory animals. Eggs of livestock species are more difficult to microinject than eggs of laboratory animals. However, competent microinjectors can reliably inflate pronuclei with DNA-containing solutions. Furthermore, integration problem occurs after the transgene is deposited. But timing of microinjection may contribute to differences in integration efficiency. It is thought that transgene integration occurs during DNA replication (2), so it would be advantageous to microinject before or during early S-phase preceding the first mitotic division. For the most part that is when laboratory animal eggs are microinjected, but microinjections are apparently performed during late S-phase or later in livestock species (for a full discussion see (63)). Efforts to inject *in vitro* fertilized bovine zygotes early have failed because of difficulties in visualizing pronuclei (K. Bondioli, personal communication and unpublished data). Efforts to synchronize microinjection and S-phase in bovine zygotes have thus far not been fruitful (24).

One way to insure that the transgene is in place before the first mitotic S-phase is to introduce the transgene at fertilization. That could be achieved by sperm-mediated gene transfer (4,38). Notwithstanding the controversy this approach has generated (8), it clearly represents an intriguing method that shows some promise (57). Accumulating evidence suggests that sperm of several species can bind transgenes (11,32,39,68) and carry the genes into oocytes where in some cases the gene persists (4,12,31). However, it appears that in almost all cases, the transgene DNA becomes rearranged or otherwise mutated by the process (Corrado Spadafora, personal communication). Another potential sperm-based delivery approach has been foretold by a pioneering study conducted by Ralph Brinster (7). In that study transplanted spermatogonial cells generated sperm capable of fertilizing oocytes and offspring were produced. If a means is found to culture, transfect and select spermatagonia with transgenes, Brinster's transplantation scheme could be used to produce transgenic animals. Others have proposed directly transfecting testes as a means of transforming sperm (56).

Retroviral-mediated gene transfer is also a potentially alternative approach for introducing transgenes into embryos with high efficiency (29,36). Though the technique solves the low integration frequency problem, it creates other inefficiencies by generating mosaic founders that may not transmit their transgene. Furthermore, retroviruses can carry only a limited amount of exogenous DNA and therefore the technique limits the size of transgenes. If cDNA based transgenes, which are relatively short, were efficiently expressed, the transgene size restriction would not be a significant problem. However, many cDNA based gene constructs are poorly expressed in transgenic animals (66).

Selection of transgenic embryos.

With no obvious or immediate solution for improving integration frequency, what else can be done to increase efficiency of producing transgenic livestock? One of the most widely discussed approaches is selection of transgenic embryos before they are transferred to recipients (1,14,35,46). If transgenic preimplantation embryos can be identified by analyzing embryo biopsies with the polymerase chain reaction (PCR), the number of recipients required could be greatly reduced. For example in Dr. Bondioli's study ((30), Table 1), 1,018 bovine embryos were transferred into over 1000 cows resulting in seven transgenic calves and fetuses. If embryo selection had been possible, fewer than 20 recipients would have been required. Unfortunately, mounting evidence suggests that this approach will not work. In two very similar studies (10,14) microinjected mouse embryos were cultured to the 8-cell stage, and blastomeres were isolated and analyzed for the transgene by PCR. In our study (10) none of the 8-cell embryos had transgenes in more than 4 blastomeres. We speculate that immediately upon microinjection, transgene copies join to form multi-copy circular arrays. One of these arrays may eventually become integrated, while the non-integrated arrays segregate as daughter blastomeres are formed. If integration occurs after the one-cell stage, some blastomeres may not contain an array, even though the embryo is transgenic. The converse is also possible (all blastomeres acquire arrays but none integrate). Analysis of embryo biopsies could therefore be misleading.

Another scheme for selecting transgenic embryos before transfer is based on expression of a selectable marker-containing transgene. The preliminary results from two recent studies (3,60) appear to be promising. In both studies, transgenes containing a neomycin resistance gene (neo) were microinjected into pronuclei of mice (60) or bovine (3) embryos. The embryos were then cultured in the presence of G418, a neomycin analog, in the hope of killing embryos that did not express the neo gene. Because this approach is based on gene expression and because transgenes can be expressed without being integrated, embryos containing unintegrated copies of the transgene could survive the selection process. However, since G418 interferes with protein synthesis, the blastomeres that expressed the neo gene would have a developmental advantage over those that did not. Therefore, the blastomeres expressing the neo gene might divide more rapidly and have a higher probability of participating in the formation of the inner cell mass (66). Further studies will have to be conducted to determine if this scheme has merit.

In The Future

The tools for gene transfer are in hand, albeit the process is inefficient. Over the next decade, bioreactor and xenograft industries will mature and useful new products will be marketed. The value of possible products will drive the technology as funding for basic research from conventional sources becomes increasingly limited. Researchers will need to develop a better understanding of how mammalian genes are controlled, and identify key genes in regulatory pathways of

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families (solid line) and acute families (dotted line). The peak multipoint lod score for chronic SMA is 9.03, and the peak lod score for acute SMA is 2.02. Pairwise lod scores for chronic and acute SMA families versus four markers located in the middle of the linkage region are shown in Table 1. The maximum two-point lod score for chronic families is 8.43 at a recombination fraction of 2% with marker D5S6, and 1.71 for acute families at a recombination fraction of 2% with marker D5S78.

Application of the HOMOG program[13] to the multipoint lod scores of the families with chronic SMA gave no evidence for heterogeneity among these families. Although the power of homogeneity tests can be lower in recessive families than in larger families with dominant diseases, the absence of evidence for heterogeneity led us to adopt the most parsimonious solution of assuming homogeneity. The confidence interval for the location of the gene for chronic SMA is 11 centimorgans (cM) wide and spans a region 2 cM proximal of locus D5S6 to a point 4 cM proximal of locus D5S78 (note arrows in Fig. 1). For families with acute SMA, the maximum lod score of 2.02 indicates that a gene responsible for this disease maps to the same general area. The best estimate for the location of the acute SMA locus is 15 cM distal to the estimated position of the locus for chronic SMA.

Our data indicate that clinically heterogeneous forms of chronic childhood SMA (type II or intermediate form and type III or Kugelberg-Welander or mild form) map to a single locus on chromosome 5q. The chronic forms of childhood-onset SMA, therefore, are likely to occur as the result of allelic heterogeneity, similar to the case for Duchenne- and Becker-type dystrophies[15]. It is interesting that our data indicate that acute childhood SMA (type I or Werdnig-Hoffmann or infantile SMA or severe SMA) map to the same, or a closely linked, locus on 5q. Other informative acute families must be analysed to confirm the linkage of this form of SMA and to evaluate the associated map location relative to that of chronic SMA. Also, other chronic families must be analysed to further assess the possible occurrence of nonallelic heterogeneity. It will be interesting to determine whether adult-onset and dominantly inherited cases of SMA similarly map to chromosome 5q. The gene encoding hexosaminidase B maps between markers D5S39 and D5S78 (refs 16, 17). Deficiencies in both the α- and β-subunit of this enzyme have been associated with chronic cases of SMA[18,19]. We are investigating whether this gene is a candidate for an SMA mutation. □

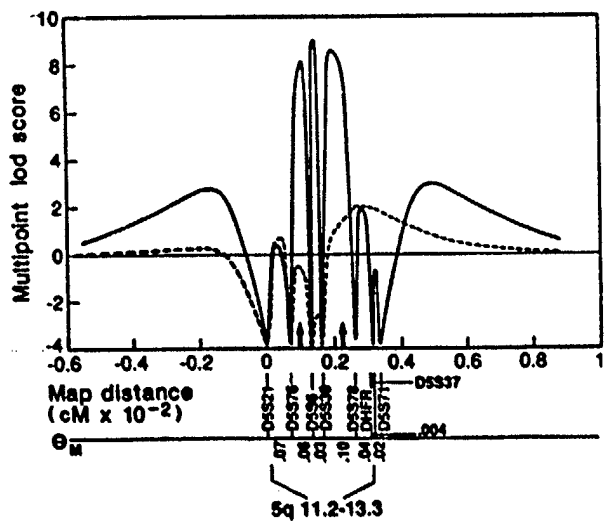

FIG. 1 Multipoint linkage analysis of the SMA disease locus with eight DNA markers spanning ~30 cM, including 5q11.2–5q13.3 (refs 16, 21). Analysis of seven chronic families (solid line) and six acute families (dotted line). Three chronic families each consists of four affected children and 8–12 unaffected sibs. Four chronic families each have three affected children and 0–4 unaffected sibs. The acute families, collected over a 3-year period, include one family with three affected children (trizygotic triplets), four families with two affected children, and one family with one affected and two unaffected sibs. Recombination fractions (θ_M) between DNA markers were calculated from published map distances[16]. Marker loci D5S6, D5S39, D5S78 and DHFR map to 5q11.2–13.3 (ref. 21). For the female-to-male distance ratio we used the published value of 1.6 as being appropriate for this area of the genome[22]. Multipoint lod scores were obtained by five-point analysis in all families, except one for which, for reasons of computational efficiency, three-point lod scores had to be calculated. The computer program used was LINKMAP of the LINKAGE package[20]. The confidence interval for chronic families (defined as points on the map with lod scores $\geq Z_{max}-1$ where Z_{max} is the value of $Z(\theta)$ at the maximum likelihood estimate of θ) spans an 11-cM region marked by arrows at map positions 0.11 and 0.22.

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Fulminant hypertension in transgenic rats harbouring the mouse *Ren-2* gene

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PRIMARY hypertension is a polygenic condition in which blood pressure is enigmatically elevated; it remains a leading cause of cardiovascular disease and death due to cerebral haemorrhage, cardiac failure and kidney disease. The genes for several of the proteins involved in blood pressure homeostasis have been cloned and characterized[1–8], including those of the renin–angiotensin system, which plays a central part in blood pressure control[9–10]. Here we describe the introduction of the mouse *Ren-2* renin gene[3,11–13] into the genome of the rat and demonstrate that expression of this gene causes severe hypertension. These transgenic animals represent a model for hypertension in which the genetic basis for the disease is known. Further, as the transgenic animals do not overexpress active renin in the kidney and have low levels of active renin in their plasma, they also provide a new model for low-renin hypertension.

We chose the mouse *Ren-2* renin gene for introduction into the rat germline because it had already been characterized in transgenic mice and because we expected it to be highly

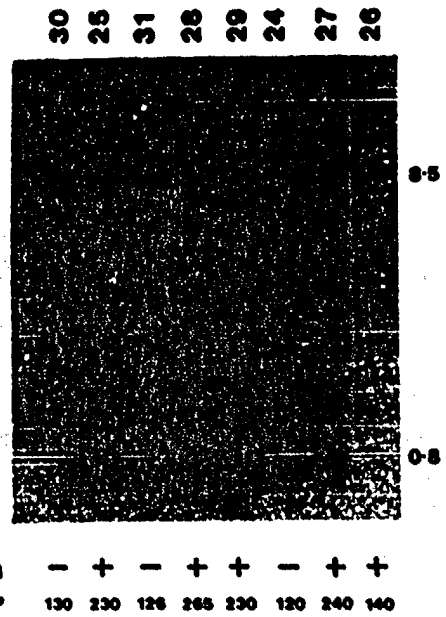


FIG. 1 Southern blot identifying animals carrying the DBA/2 *Ren-2* gene. The identification numbers of potential founder animals are shown above the corresponding lane and the positions of the *Ren-2*-specific 8.5-kb and 0.8-kb restriction fragments are indicated to the right. Transgenic (TG) positive and negative animals are indicated by symbols under the corresponding lane, together with the systolic blood pressure (BP, in mm Hg) of each animal at the age of 10 weeks.
METHODS. DNA preparation: DNA was prepared from tail biopsies and digested with *Pvu*II. After electrophoresis on a 0.8% agarose gel, samples were Southern-blotted and hybridized with a ^{32}P-labelled dCTP 300-bp probe derived from the renin complementary DNA clone pDD1D2[17] and labelled by random priming[18]. Preparation of transgenic animals: DNA was prepared for microinjection by digestion of the cosmid clone cosDBA-1 (ref. 17) with *Xho*I, and subsequent isolation of the 24-kb *Xho*I fragment containing the *Ren-2* gene on a 10–20% sucrose gradient in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM sodium acetate. Fractions containing the required fragment were pooled and recovered by ethanol-precipitation before being centrifuged on a CsCl gradient[19]. DNA was diluted to a final concentration of 1 μg ml^{-1} in injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), and stored in aliquots at −20 °C before use. Fertilized eggs were derived from a cross between Sprague-Dawley female and WKY male rats after superovulation of immature females (at 4 weeks old) according to the procedure of Armstrong *et al.*[20]. Eggs were cultured, microinjected, and re-implanted as described for the mouse[19]. Rats were all bred in our own facilities.

expressed in certain tissues[14]; also, injection of purified mouse submandibular gland (SMG) renin (encoded by *Ren-2*) into rats leads to a significant and sustained increase in blood pressure[15]. Fertilized rat eggs were microinjected with a linear DNA fragment containing the entire DBA/2J *Ren-2* gene, including 5.3 and 9.5 kilobases (kb) of 5′ and 3′ flanking sequence, respectively[14]. From 37 eggs implanted, there were eight progeny, of which five carried the transgene (Fig. 1). Four of the founders were bred successfully and three of them (TGRmRen2, numbers 25, 26 and 27) transmitted the transgene to their progeny. At ten weeks of age and before breeding, the blood pressure of the founder animals was measured. For four of the transgenic animals it was in the range 230–265 mm Hg, but was 120–130 mm Hg in the transgene-negative litter-mates (Fig. 1). Breeding of TGRmRen2 female 26, who was not hypertensive, revealed her to be mosaic for a transgene insertion site, the inheritance of which segregated with hypertension in the blood pressure range indicated (data not shown). The phenotype is therefore independent of the transgene insertion site and is not due to a fortuitous mutation associated with the integration event.

Analysis of the transgenic line established from TGRmRen2 male 27 revealed that, without exception, progeny inheriting the transgene also had the hypertensive phenotype. Both male and female animals of this line developed hypertension rapidly, beginning at four weeks of age and reaching a maximum by nine weeks (Fig. 2*a*). Pharmacological intervention to reduce blood pressure took the form of treating the animals with 10 mg kg^{-1} per day of the converting enzyme inhibitor, captopril; this inhibits the conversion of angiotensin I to angiotensin II. This low dose, given daily in the drinking water, was sufficient to reduce the blood pressure of the hypertensive transgenic rats reproducibly by 40–60 mm Hg (Fig. 2*b*), indicating that the hypertension is largely dependent on the conversion of angiotensin I to angiotensin II.

Northern blot analysis showed that the concentration of renin transcripts was high in the adrenal glands of the transgenic animals (Fig. 3*a*). In addition, renin transcripts were detectable in testis, coagulation gland, thymus and small intestine in transgene-positive animals, but not in control transgene-negative littermates (data not shown). These additional sites represent tissues in which renin is naturally expressed in the mouse. Renin messenger RNA was not observed in the SMG, a result that could reflect the absence of essential *trans*-acting factors in this tissue as the endogenous rat renin gene is not expressed in the SMG (ref. 16). An RNase protection assay using a *Ren-2*-specific probe confirmed that the renin transcripts in the adrenal gland were exclusively of *Ren-2* origin and that *Ren-2* transcripts were present in the kidneys of transgene-positive animals (Fig. 3*b*).

No evidence was found for altered plasma angiotensinogen levels, but plasma renin activity and angiotensin I were significantly lower in transgenic animals than in the controls (Fig. 4*b*–*e*). The amount of angiotensin II was also less than in the

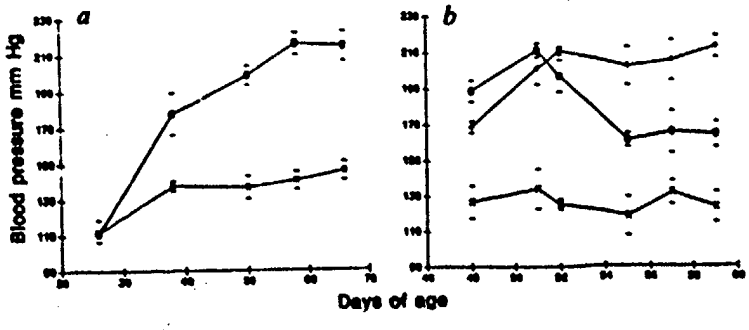


FIG. 2 *a*, Development of blood pressure with age. Each point represents the mean of 7 (transgenic, circles) or 5 (control, crosses) animals and standard errors are indicated above and below each data point. *b*, Effect of converting enzyme inhibitor (CEI) on blood pressure. Each point represents the mean of 3 animals and standard errors are indicated above and below each data point. +, TGRmRen2 L27 rats having no treatment; O, TGRmRen2 L27 rats receiving CEI; ×, control rats receiving CEI.
METHODS. Blood pressure was determined by tail plethysmography under light ether anaesthesia as described[21]. Animals under converting enzyme inhibitor treatment were given captopril (10 mg kg^{-1} per day) in their drinking water. Captopril treatment started at day 51.

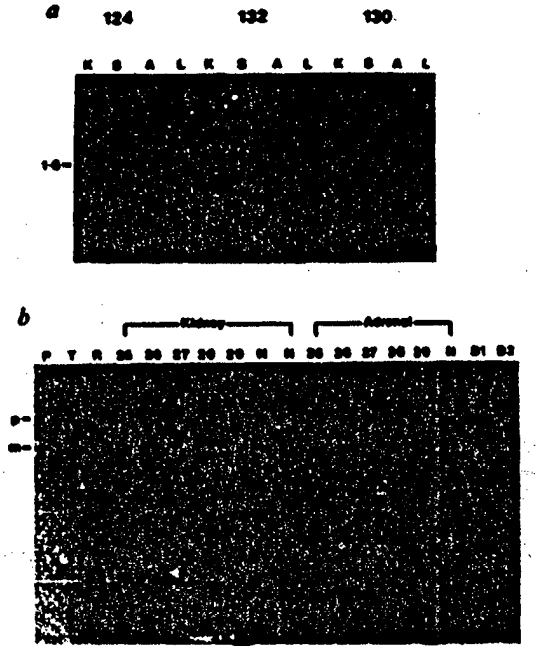


FIG. 3 Northern blot and RNase protection assay. *a*, Northern blot of RNA isolated from the kidney (K); SMG (S); adrenal gland (A); and liver (L) of transgene-positive (124 and 130) and transgene-negative (132) male rats. The size of the hybridizing RNA is indicated in kb. With the exception of the adrenal gland (5 μg), 40 μg total RNA was used for each sample. *b*, RNase protection of kidney and adrenal gland RNA from transgenic rats (numbers 25–29) and control littermates (26). The following controls are included: P, undigested probe; T, transfer RNA (9 μg); R, rat kidney RNA (20 μg); D1 and D2, mouse (DBA/2J) kidney RNA (20 μg and 40 μg, respectively). The positions of the undigested 244-nucleotide probe (p) and the 224-nucleotide mouse-specific protected fragment (m) are indicated.

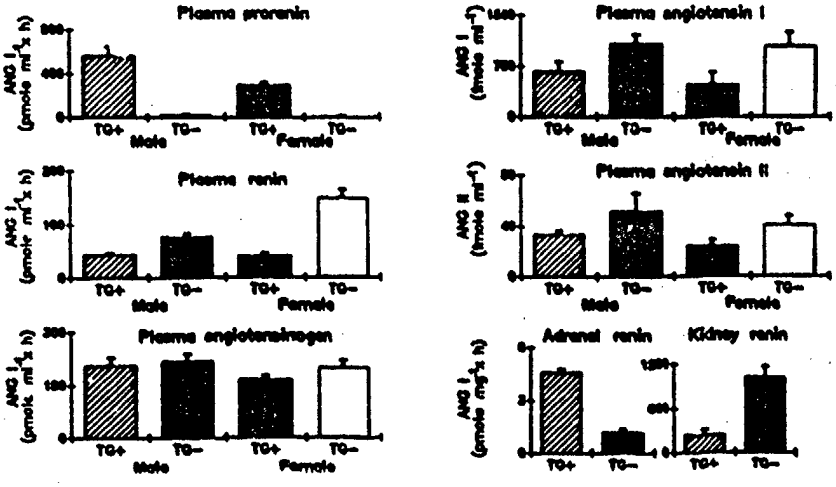
METHODS. Preparation of RNA: Total RNA was isolated from mature rats as previously described[3] or by homogenization in guanidine isothiocyanate.[22] Northern blot analysis: Northern blots were prepared and hybridized as previously described[23] with a ^{32}P-labelled renin cDNA probe (pDD1D2) by random priming[18] and washed with 0.1 × SSC, 0.1% SDS at 65 °C. RNase protection assay: ^{32}P-labelled RNA transcripts were prepared by transcription of a 244-nucleotide antisense RNA from the plasmid pSLM (ref. 15) using SP6 RNA polymerase. This transcript comprised 224 nucleotides of *Ren-2* antisense RNA and 20 nucleotides of vector-encoded sequence. Samples were dissolved in 30 μl 80% formamide, containing 40 mM PIPES, 400 mM NaCl, 1 mM EDTA and 200,000 c.p.m. of the gel-purified transcript, denatured at 100 °C for 1 min and incubated at 45 °C for 20 h. RNase digestion was performed in 300 μl buffer containing 40 μg ml^{-1} RNase A (Sigma) and 2 μg ml^{-1} RNase T1 (Calbiochem) for 45 min at 37 °C. After digestion with proteinase K, samples were electrophoresed on denaturing 5% polyacrylamide gels.

controls but the difference was not statistically significant. Determination of prorenin showed it to be raised in the plasma of transgenic animals (Fig. 4*a*), but the functional significance of this finding is unclear. Adrenal glands of the transgenic animals contained significantly increased renin concentrations (Fig. 4*f*). No evidence was found for the storage of renin in this tissue, so the large difference between renin mRNA levels and enzyme activity may reflect a constitutive secretion of *Ren-2*-derived renin from the adrenal glands. By contrast, kidney tissue from transgenic animals contained only 20–25% of the renin activity of the controls, which is consistent with immunocytochemical and ultrastructural data showing a reduction in renin storage granules in the juxtaglomerular apparatus (S. Bachmann *et al.*, manuscript in preparation) and suggests that renin expression is subject to translational or post-translational control. Preliminary studies on isolated kidney show that renin secretion is reduced and that there are no other abnormalities of renal function (K. Munter, personal communication).

Although we have defined a genetic basis for this transgenic hypertensive rat model, the mechanism responsible for elevating blood pressure remains to be established. The hypertension is clearly not due to overexpression of renin in the kidney, and the suppression of active renin in the kidney and in the plasma is probably a result of an already elevated blood pressure in young animals, pressure-mediated renin suppression being a well known phenomenon. The increased plasma prorenin probably originates, at least in part, from the adrenal gland, but the ovary, vascular tissue and other sources of prorenin should also be considered. Any role of prorenin in hypertension still awaits investigation, but in this respect it is interesting that prorenin is raised and still persists after nephrectomy in hypertensive patients, confirming that its origin is extra-renal. At this stage, the most likely explanation for the high blood pressure in TGRmRen2 rats is a stimulated renin–angiotensin system in the adrenal gland, with the consequent overproduction of steroid hormones. This is in keeping with our preliminary data on

FIG. 4 Determination of plasma and tissue renin-angiotensin system components. Values represent the mean and standard error of 7 animals for each determination, with the exception of the kidney and adrenal gland renin values (3 animals). Statistical analysis by ANOVA showed the following significance values: prorenin, $P < 0.05$ between the transgenic animals and the corresponding controls; renin, $P < 0.005$ between the transgenic animals and the corresponding controls; angiotensin I, $P < 0.05$ between the transgenic animals and the corresponding controls; tissue renin, $P < 0.01$ for the adrenal gland and $P < 0.005$ for the kidney.

METHODS. Concentrations of angiotensinogen, angiotensin I, angiotensin II and renin were determined as described[24–26]. Prorenin levels were calculated by subtraction of renin activity from total plasma renin activity determined after trypsin activation[26].



elevated urinary aldosterone excretion in male TGRmRen2 rats (15.4 ± 2.26 ng per 24 h) compared with controls (8.97 ± 1.06 ng per 24 h). These animals will enable us to study normal or low plasma renin hypertension and have shown us that renin can participate in the genesis of hypertension in a more subtle way than previously supposed. The construction of transgenic rats will therefore provide new opportunities for research into cardiovascular mechanisms. □

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Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses

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IN mammalian muscle, the subunit composition of the nicotinic acetylcholine receptor (AChR) and the distribution of AChRs along the fibre are developmentally regulated. In fetal muscle, AChRs are distributed over the entire fibre length whereas in adult fibres they are concentrated at the end-plate[1]. We have used *in situ* hybridization techniques to measure the development of the synaptic localization of the messenger RNAs (mRNAs) encoding the α-subunit and the ε-subunit of the rat muscle AChR. The α-subunit is present in both fetal and adult muscle, whereas the ε-subunit appears postnatally and specifies the mature AChR subtype[2–4]. The synaptic localization of α-subunit mRNA in adult fibres may arise from the selective down-regulation of constitutively expressed mRNA from extrasynaptic fibre segments. In contrast, ε-subunit mRNA appears locally at the site of neuromuscular contact and its accumulation at the end-plate is not dependent on the continued presence of the nerve terminal very early during synapse formation. This suggests that ε-subunit mRNA expression is induced locally via a signal which is restricted to the end-plate region and is dependent on the presence of the nerve only during a short period of early neuromuscular contact. Evidently, several mechanisms operate to confine AChR mRNAs to the adult end-plate region, and the levels of α-subunit and ε-subunit mRNAs depend on these mechanisms to differing degrees.

Hybridization of longitudinal sections of adult rat soleus muscle with ε- and α-subunit-specific antisense complementary RNA (cRNA) probes revealed strong hybridization signals at sites that had been previously identified as end-plates by staining for acetylcholinesterase (AChE). Figure 1*a* shows the end-plate region of a muscle stained for AChE. Subsequent hybridization with the ε-subunit-specific antisense probe showed a strong signal at the site where the AChE stain had been (Fig. 1*b*). After a brief exposure, groups of grains could be resolved above individual synaptic nuclei (Fig. 1*c*); no hybridization was observed outside end-plate regions. When sections were incubated with ε-subunit-specific sense probes, no hybridization could be detected (data not shown). These observations suggest that autoradiographic grain clusters reflect locally increased ε-subunit mRNA levels below the end-plate membranes. Similar results were obtained after hybridization with α-subunit-specific antisense (Fig. 1*d*, *e*) and sense probes and confirm the synaptic localization of α-subunit mRNA in rat muscle, as observed previously using northern blot analysis[3]. In some fibres, a small signal was observed above nuclei in the perijunctional region of the muscle fibres (Fig. 1*e*).

Previous northern blot analysis of AChR-specific mRNAs in neonatal rat muscle indicated that ε-subunit mRNA is barely detectable at birth but that levels increase rapidly during the first 2 weeks of postnatal development[4]. To determine whether this increase in ε-subunit mRNA is restricted to the end-plate region and therefore would be induced focally by the nerve, or whether the increase is more general, involving the entire fibre, we hybridized triceps muscle from rats of different postnatal ages with an ε-subunit mRNA-specific cRNA probe. Figure 2*a* shows the localization of AChE and autoradiographs of longitudinally sectioned muscle (*b*–*d*). At postnatal day 1, no hybridization signal could be detected either synaptically or extrasynaptically (Fig. 2*b*). In dark-field microscopy, some of the synaptic sites revealed a weak accumulation of grains (data not shown). However, on postnatal days 5, 9 (data not shown) and 12, an increasingly stronger signal was seen (Fig. 2*c*) that always coincided with the AChE-stained synaptic sites. Thus, the postnatal appearance of ε-subunit mRNA is restricted to the end-plate region from the earliest stages of synapse development and therefore must be induced by the nerve-muscle contact. As in adult muscle, hybridization signals in postnatal day-12 muscles were clearly associated with individual nuclei, as shown in Fig. 3. However, given the high density of nuclei from various cell types, unequivocal attribution to subneural nuclei was not always possible.

In contrast, total α-subunit mRNA remained at a plateau level during the first 12 postnatal days[4]. During this period, the α-subunit mRNA was detected throughout the fibre, in both the synaptic and extrasynaptic fibre segments (Fig. 2*f*, *g*). Although there were more grains at the synaptic sites, they were more widely distributed than those obtained upon hybridization with the ε-subunit mRNA specific probe. Moreover, the hybridization signal was also observed outside the myofibre bundles above unfused cells.

The level of total muscle ε-subunit mRNA increases almost normally in neonatal muscle denervated shortly after birth[4], indicating that only the brief, prenatal nerve-muscle contact is necessary to induce ε-subunit mRNA synthesis. We have investigated whether the ε-subunit mRNA still appears focally at the

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Spontaneous Inflammatory Disease in Transgenic Rats Expressing HLA-B27 and Human β_2m: An Animal Model of HLA-B27-Associated Human Disorders

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Summary

Humans who have inherited the human class I major histocompatibility allele HLA-B27 have a markedly increased risk of developing the multi-organ system diseases termed spondyloarthropathies. To investigate the role of B27 in these disorders, we introduced the B27 and human β_2-microglobulin genes into rats, a species known to be quite susceptible to experimentally induced inflammatory disease. Rats from one transgenic line spontaneously developed inflammatory disease involving the gastrointestinal tract, peripheral and vertebral joints, male genital tract, skin, nails, and heart. This pattern of organ system involvement showed a striking resemblance to the B27-associated human disorders. These results establish that B27 plays a central role in the pathogenesis of the multi-organ system processes of the spondyloarthropathies. Elucidation of the role of B27 should be facilitated by this transgenic model.

Introduction

Class I major histocompatibility (MHC) gene products are polymorphic 44,000 M_r glycoproteins expressed on cell surfaces in noncovalent association with the nonpolymorphic 12,000 M_r light chain β_2-microglobulin (Klein, 1986). Among class I MHC molecules, HLA-B27, a serologically defined allele of the human *HLA-B* locus, is of particular interest because it is uniquely associated with a group of relatively common inflammatory disorders. The strongest association is seen with primary ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system: ~90% of affected individuals have inherited the B27 allele in comparison with only ~7% of Caucasians in the general population (Brewerton et al., 1973; Schlosstein et al., 1973; Tiwari and Terasaki, 1985). An important association also exists between HLA-B27 and reactive arthritis, in which certain microbial infections of the gastrointestinal or genitourinary tracts trigger inflammation in joints and other tissues (Toivanen and Toivanen, 1988). A summary of the major disorders associated with HLA-B27 is presented in Table 1.

The B27-associated diseases are classified as rheumatic disorders because of the prominence of musculoskeletal manifestations. Nonetheless, all of these diseases can involve multiple organ systems, particularly the gastrointestinal tract, genitourinary tract, skin, eye, and heart. Because of the overlap among these diseases with regard to epidemiology, clinical manifestations, and anatomic pathology, they were recognized as a distinct cluster of interrelated diseases, termed spondyloarthropathies, even before the common genetic marker of B27 was identified (Moll et al., 1974). Thus it has long been speculated that a common pathogenetic mechanism might underlie the association of B27 with this heterogeneous group of disorders. Despite extensive investigation, however, the etiology and pathogenesis of these diseases have remained obscure, and the basis for the association with B27 has not been established.

In an attempt to develop an animal model of B27-associated disease, we (Taurog et al., 1988a) and others (Krimpenfort et al., 1987; Nickerson et al., 1990; Weiss et al., 1990) have produced transgenic mice expressing HLA-B27 and human β_2-microglobulin (hβ_2m). However, despite physiologically normal function of B27 in both hybrid and inbred mice (Kievits et al., 1987; Taurog et al., 1988a) and a reported influence of B27 on the course of an experimental bacterial infection in mice (Nickerson et al., 1990), no faithful reproduction of any of the features of B27-associated human disease has been reported in transgenic mice. These negative results raised the possibility that susceptibility to the spondyloarthropathies might not be related to the B27 gene. Alternatively, other features of the mouse may not have permitted expression of the relevant pathologic changes. We therefore sought to develop transgenic technology in rats, which are susceptible to several experimentally induced arthritic diseases that cannot be elicited in mice (Greenwald and Diamond, 1988).

In this paper, we describe the production of transgenic rats that express HLA-B27 and hβ_2m genes. We further describe a disorder spontaneously arising in these B27 transgenic rats that includes most of the features of B27-associated disease in humans.

Results

Integration of HLA-B27 and hβ_2m Genes in Inbred Rats

Fertilized one-cell rat eggs were microinjected with a solution containing both DNA fragments shown in Figure 1. The HLA-B27 gene encoding the HLA-B*2705 subtype was contained on a 6.5 kb EcoRI fragment that included 0.7 kb of 5′ flanking sequence and 2.5 kb of 3′ flanking sequence (Figure 1A). The hβ_2m gene was contained on a 15 kb SalI–PvuI fragment that included 5.2 kb of 5′ flanking sequence and 1.9 kb of 3′ flanking sequence (Figure 1B). Identification and quantitation of transgenes in the founder animals and their progeny were determined by dot-blot hybridization of genomic DNA isolated from tail bi-

Table 1. Rheumatic Diseases Associated with HLA-B27

Characteristic	Disorder				
	Ankylosing Spondylitis	Reactive Arthritis[a]	Juvenile Spondyloarthropathy	Psoriatic Arthropathy	Enteropathic Arthropathy
Sacroiliitis or spondylitis[b]	100%	<50%	<50%	20%	10%
Peripheral arthritis[c]	25%	90%	90%	95%	90%
Gastrointestinal inflammation	Common, usually asymptomatic	Common, often symptomatic	Not known	Uncommon	All
Skin and nail involvement	Rare	Most	Uncommon	All	Uncommon
Genitourinary involvement (males only)	Uncommon	Most	Uncommon	Uncommon	Rare
Eye involvement[d]	25%	Common	Common	Occasional	Occasional
Cardiac involvement	<5%	5%–10%	Not known, probably rare	Rare	Rare
Usual age of onset (years)	18–40	18–45	7–18	20–50	15–50
Sex prevalence	Males 3:1	Males 3:1[e]	Males 10:1	Equal	Equal
Type of onset	Gradual	Acute	Variable	Variable	Gradual
Role of infectious agents	Unknown	Definite Trigger	Unknown	Unknown	Unknown
Prevalence of HLA-B27[f]	>90%	60%–80%	80%	50%[g]	50%–75%[g]

Table adapted from Calin (1984); Tiwari and Terasaki (1985); Khan and van der Linden (1990); Taurog and Lipsky (1990).
[a] Includes Reiter's syndrome, classically defined as the triad of arthritis, conjunctivitis, and urethritis.
[b] Inflammation in the spine or sacroiliac joints.
[c] Inflammation in joints of the extremities.
[d] Predominantly conjunctivitis in reactive arthritis; iritis with the other disorders.
[e] Male to female ratio is 10:1 if venereally acquired; 1:1 if enteropathically acquired.
[f] Caucasians of northern European extraction only. General prevalence in this population is 6%–8%. Some variation seen in other populations, but the basic associations with HLA-B27 are seen worldwide.
[g] Frequency elevated only in those with spondylitis or sacroiliitis.

opsies. Hybridization was carried out with 5′ and 3′ flanking probes for the HLA-B27 gene (probes A and C in Figure 1A), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the hβ_2m gene (probe D in Figure 1B).

Seven LEW and four F344 rats that developed from microinjected ova showed integration of the HLA-B27 and hβ_2m genes. Of these, four LEW rats and one F344 rat showed cell surface expression of both HLA-B27 and hβ_2m, as assessed by indirect immunofluorescence of peripheral blood lymphocytes (PBLs). One additional LEW rat showed integration and expression of the B27 gene alone. Table 2 summarizes the results of the microinjection experiments.

All of the founder rats expressing the transgenes were subsequently shown to transmit the transgenes to their offspring. One of the six founders, 21-3, was found to be a mosaic, based on non-Mendelian rates of transmission and on enhanced cell surface expression in the offspring. Another founder, 21-4, a female, was shown to have two independently segregating loci of transgene integration, each locus carrying both transgenes. One line arising from this founder, inheriting a locus containing 150 copies of the B27 gene and 90 copies of the hβ_2m gene, was termed 21-4H. The other line, inheriting a locus containing six copies of the B27 gene and six copies of the hβ_2m gene, was termed 21-4L (Table 3).

Lymphocyte Cell Surface Expression of the HLA-B27 and hβ_2m Transgene Products

Expression of the transgene products was estimated by indirect immunofluorescence and flow cytometry of PBLs stained with specific monoclonal antibodies. The relative expression of B27 and hβ_2m in seven transgenic lines is shown in Table 3. To compensate for interexperiment variation, the mean channel fluorescence for each line with each antibody is expressed relative to that determined in the same experiment for PBLs of the transgenic mouse line 56-3, which expresses high levels of both B27 and hβ_2m on PBL surfaces. The highest expression of both gene products was found in the LEW lines 21-4H and 21-4L and the F344 line 33-3.

The patterns of cell surface expression of B27 and hβ_2m in the 21-4H and 21-4L lines are shown in Figures 2A and 2B. The binding of the endogenous rat class MHC I molecules (RT1) to the anti-RT1 antibody OX18 is shown in Figure 2C for both transgenic lines and the nontransgenic control. The levels of expression of B27 and hβ_2m were comparable in the two transgenic lines (Figures 2A

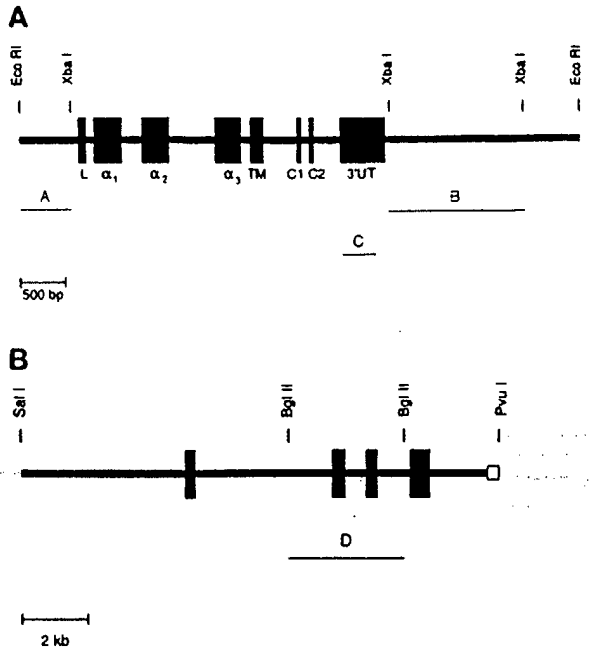

Figure 1. Genes Used for Microinjection of Fertilized Rat Eggs

(A) The HLA-B* 2705 gene (clone pE.1-B27) was contained on a 6.5 kb EcoRI fragment. Exons are indicated by boxes and labeled. Probes from the 5′ and 3′ flanking regions, labeled A and B, respectively, were used for dot-blot hybridization of genomic DNA. Probe C, from the 3′ untranslated region, was used for Northern hybridization.

(B) The hβ2m gene (clone pβ2m-13) was contained on a 15 kb SalI–PvuI fragment. Exons are indicated by boxes. The insert contained ~100 bp of the vector pEMBL9, indicated by the open box at the 3′ end. The 3.7 kb BglII fragment labeled D was used for both dot-blot hybridization of genomic DNA and for Northern hybridization.

Table 2. Production of HLA-B27 and hβ2m Transgenic Rats

			Founder			
			Integration[b]		Expression[c]	
Strain	Eggs[a]	Pups	B27	hβ2m	B27	hβ2m
LEW	348	23	8	7	5	4
F344	329	24	4	4	1	1

[a] Number of eggs injected and transferred to pseudopregnant recipients.

[b] Transgenic animals were identified by dot-blot analysis of DNA isolated from tails.

[c] Cell surface expression was assessed by indirect immunofluorescence and flow cytometry of PBLs.

and 2B), and in both lines the expression of the endogenous RT1 class I molecules appeared to be reduced in comparison with the nontransgenic control (Figure 2C).

Immunologic Function of the HLA-B27 Transgene

To assess T cell recognition of the B27 transgene product as a class I MHC antigen, primary grafts of B27 transgenic LEW rat skin were placed on nontransgenic LEW rats, and spleen cells from the recipient rats were subsequently tested for B27-specific cytotoxicity. As shown in Table 4,

Table 3. Copy Number and Cell Surface Expression of HLA-B27 and hβ2m in Transgenic Rat Lines

	Gene (Copy/Cell)[a]		Cell Surface Expression (Relative MCF)[b]	
Line	B27	hβ2m	B27	hβ2m
21-2	1	1	0.09	0.06
21-3	20	15	0.30	0.29
21-4L	6	6	0.74	0.42
21-4H	150	90	0.51	0.42
25-1	1	0	0.15	0.00
25-6	7	7	0.42	0.27
33-3	55	66	1.00	0.76

[a] Gene copy number was estimated by quantitative dot hybridization on DNA isolated from tails using probes specific for each transgene (see Figure 1A).

[b] Mean channel fluorescence (MCF) with antibodies to HLA-B (B1.23.2) or hβ2m (BBM.1) of PBLs from transgenic rats, relative to simultaneously determined MCF of PBLs from the B27/hβ2m transgenic mouse line 56-3. All data are from progeny of founders to eliminate influence of mosaicism.

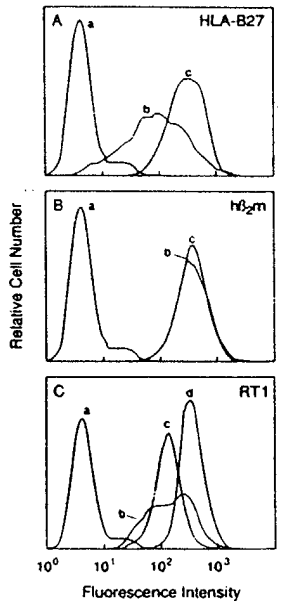

Figure 2. Comparison of Cell Surface Expression of HLA-B27, hβ2m, and the Endogenous RT1 Class I MHC Molecules in 21-4H, 21-4L, and Nontransgenic Rats

Peripheral blood mononuclear cells were incubated with saturating concentrations of monoclonal antibodies and fluorescein-labeled second antibodies and then analyzed by flow cytometry, as described in Experimental Procedures. The results demonstrate that cell surface expression of both transgenes was at least as high in the clinically normal 21-4L line as in the disease-prone 21-4H line and that endogenous RT1 expression appeared lower in the transgenic rats than in the nontransgenic control. Sources of cell populations were nontransgenic LEW stained with negative control antibody (a), 21-4H (b), 21-4L (c), and nontransgenic LEW stained with anti-RT1 antibody (d). Monoclonal antibodies were anti-HLA-B27 (B.1.23.2) (A), anti-hβ2m (BBM.1) (B), and anti-RT1 class I (OX18) (C).

Table 4. Cell-Mediated Cytotoxicity against HLA-B27

Effector Cells		Effector to	% Cytoxicity of Target Cells	
Donor	Recipient	Target Ratio	$B27^+h\beta_2m^+$	$B27^-h\beta_2m^+$
Experiment 1				
21-4L	LEW	100	34	15
		50	38	8
		20	36	3
LEW	LEW	100	19	13
		50	14	7
		20	5	1
Experiment 2				
21-4H	LEW	100	18	7
		50	8	4
		20	3	2
LEW	LEW	100	3	3
		50	1	2
		20	0	1

Spleen cells from LEW rats grafted 7 days earlier with skin from either 21-4 transgenic or normal LEW donors were incubated at the indicated effector target ratios with ^{51}Cr-labeled murine L cell targets expressing either $h\beta_2m$ alone or $h\beta_2m$ and HLA-B27. Incubation times: experiment 1, 6 hr; experiment 2, 4 hr. SD <15% in experiment 1 and <10% in experiment 2.

spleen cells from nontransgenic LEW rats receiving grafts from either 21-4H or 21-4L donors showed significantly higher lytic activity against L cell targets transfected with the B27 gene than against otherwise identical targets lacking this gene. Lytic activity was also higher in recipients of transgenic grafts than in recipients of control nontransgenic syngeneic grafts. These results indicate that the B27 transgene product is recognized in a conventional manner by allogeneically primed cytolytic T cells.

Inflammatory Disease in the 21-4H Line: Clinical and Histologic Findings

Gastrointestinal Tract

Overt disease appeared in all of the rats bearing the 21-4H transgene locus that survived past 10 weeks of age. This cohort consisted of 14 males and 9 females. The most common and persistent finding was diarrhea, manifested by frequent, voluminous, often watery stools. Diarrhea was observed in all 23 animals, with equal persistence and severity in the two sexes. Histologically, the gastrointestinal disease was manifested by chronic inflammation involving the stomach and small and large intestine (Figure 3). The distribution and severity of the lesions varied, the colon being the most consistently and prominently affected site. Less frequently, gastric lesions predominated. In all sites, the inflammatory cells consisted primarily of large and small lymphocytes, plasma cells, and smaller numbers of eosinophils. Although the inflammatory response remained primarily in the lamina propria, in the most severely affected regions it extended into the submucosa. Lymphocytes were commonly aggregated into small hyperplastic lymphoid foci, especially in the colon and ileum.

In the intestinal lesions, hyperplasia of crypt epithelial cells replaced mucus-secreting cells and increased the depth of the crypts (Figures 3D and 3F). Hyperplastic crypt cells showed regenerative atypia and a marked increase in mitotic activity. Destruction of crypts and/or the formation of crypt abscesses was uncommon and seen only in the most inflamed areas.

The gastric lesions generally consisted of widely scattered inflammatory foci in the lamina propria and submucosa, but in more severe lesions inflammation was much more extensive, and inflammatory cells accumulated in ectatic glands. The proliferation of mucus-neck cells resulted in marked reduction in the number of parietal cells (Figure 3B).

That the gastrointestinal inflammation did not result from a contagious pathogen was suggested by four pieces of evidence. Stool cultures for aerobic bacteria yielded only normal fecal flora. Furthermore, rats of the 21-4L line and nontransgenic LEW rats were housed for long periods in the same cages with affected 21-4H rats without showing any diarrhea or other signs of illness. In addition, the histology of the gastrointestinal tract of the affected 21-4H rats was not consistent with any known infectious process. Finally, diarrhea has also appeared in six out of seven transgenic rats of the 33-3 line past the age of 2 months, and not in their nontransgenic littermates.

Peripheral and Axial Joints

Peripheral arthritis was observed in 10 of 14 21-4H males and in 1 of 9 21-4H females. This was manifested in most cases by swelling, erythema, and tenderness of the tarsal joints of one or both hindlimbs (Figure 4B). In a few animals the carpal joints or digits were also inflamed (Figure 4D). The arthritis persisted from a few days to several weeks, and in some cases showed an undulating pattern of remission and exacerbation.

Histologically, large accumulations of neutrophils were present in the joint space. The synovium was hyperplastic, edematous and infiltrated with large numbers of lymphocytes, plasma cells, and neutrophils, with neutrophils predominating in the most active lesions (Figure 6B). There was marked pannus formation that eroded the bone at the synovial recess, invading and destroying the articular cartilage. Where the articular cartilage on adjacent joint surfaces was completely replaced by pannus, fibrous ankylosis occurred. Reactive bone formed small osteophytes along the diaphyses, and foci of metaplastic bone were seen within the fibrotic joint capsule. Chronic inflammation extended from the joint capsule to involve adjacent ligaments and tendons. Despite extensive joint destruction evident histologically, resolution generally occurred with preservation of mobility in the large joints.

Vertebral joints from two tails of 21-4H rats were examined histologically, and both revealed inflammatory changes at the outer aspects of the annulus fibrosus and its attachment to the vertebral endplate (Figure 6D). The inflammatory cells consisted of lymphocytes and small numbers of plasma cells mixed with active fibroblasts. There was active bone resorption at the insertion of the annulus and the adjacent periosteum was reactive.

Skin and Nails

Several animals of both sexes developed grossly evident

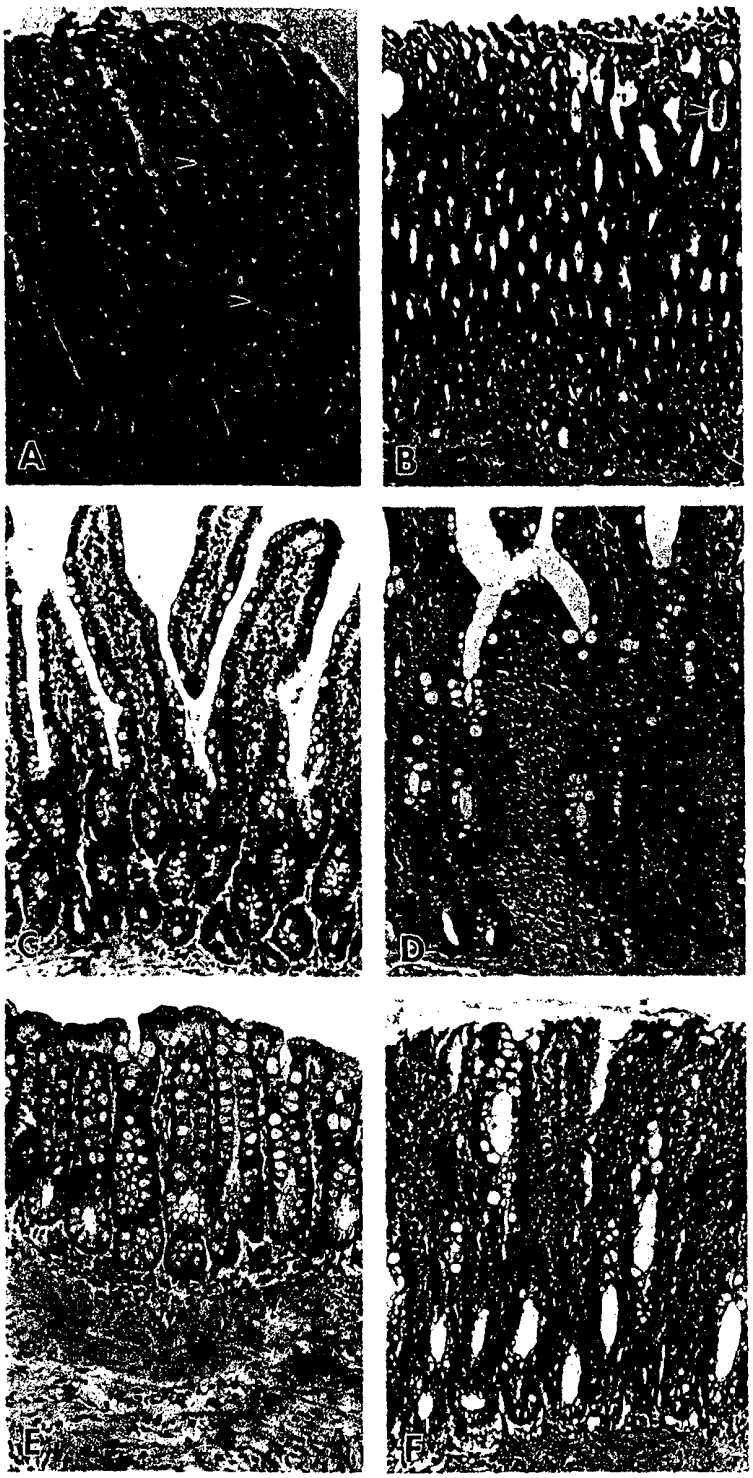

Figure 3. Gastrointestinal Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal stomach. Arrowheads indicate typical parietal cells (81.25×).

(B) Stomach of a 3-month-old 21-4H male, showing chronic gastritis, with numerous dilated pits and glands (asterisks). A microabscess is present in one dilated gland (arrowhead). Hyperplasia of the mucus-neck cells has largely replaced the parietal cells, and an inflammatory infiltrate is present throughout the lamina propria (65×).

(C) Normal ileum (84.5×).

(D) Ileum of a 3-month-old 21-4H male, showing chronic enteritis. The depth of the crypts is increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (84.5×).

(E) Normal colon (97.5×).

(F) Colon of a 3-month-old 21-4H male, showing chronic colitis. The depth of the crypts is markedly increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (97.5×).

changes in the tail skin and/or dramatic hyperkeratosis and dystrophy of the nails on all four extremities (Figures 5B and 5D). Histologically, in the tail lesions the epidermis was massively thickened by psoriasiform hyperplasia (Figure 6F). The rete ridges were regular and thickened at the base. Exocytosis of lymphocytes and neutrophils was common, with these cells accumulating in spongiotic foci in the epidermis, in the superficial parakeratotic crust, or around degenerated, necrotic keratinocytes. Diffuse orthokeratotic hyperkeratosis was prominent. The superfi-

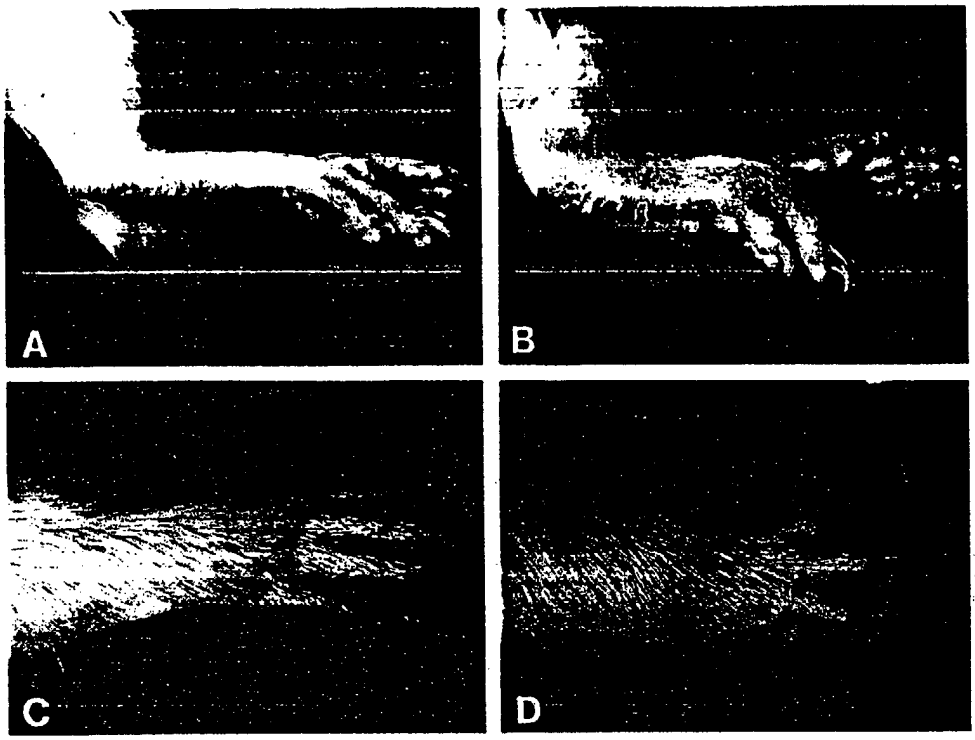

Figure 4. Peripheral Joint Gross Pathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal distal hindlimb.

(B) Distal hindlimb of a 6-month-old 21-4H male showing swelling and erythema.

(C) Normal distal forelimb.

(D) Distal forelimb of a 4-month-old 21-4H male showing swelling and erythema surrounding the carpal joint.

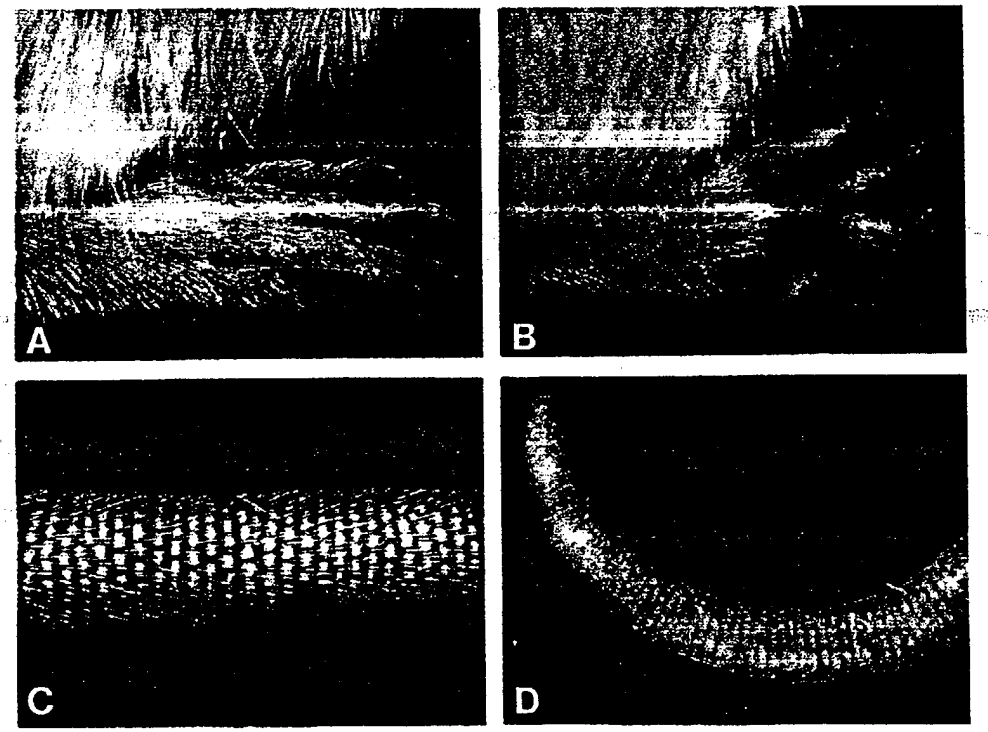

Figure 5. Nail and Skin Gross Pathology of 21-4H Rats

(A) Normal hindlimb digits and nails.

(B) Hindlimb digits and nails of a 3½-month-old 21-4 male, showing hyperkeratosis and dystrophy of the nails and alopecia over the digits.

(C) Normal tail.

(D) Tail of a 3½-month-old 21-4 male (same as in [B]), showing edema, alopecia, flaking, and masking of the normal ridged pattern.

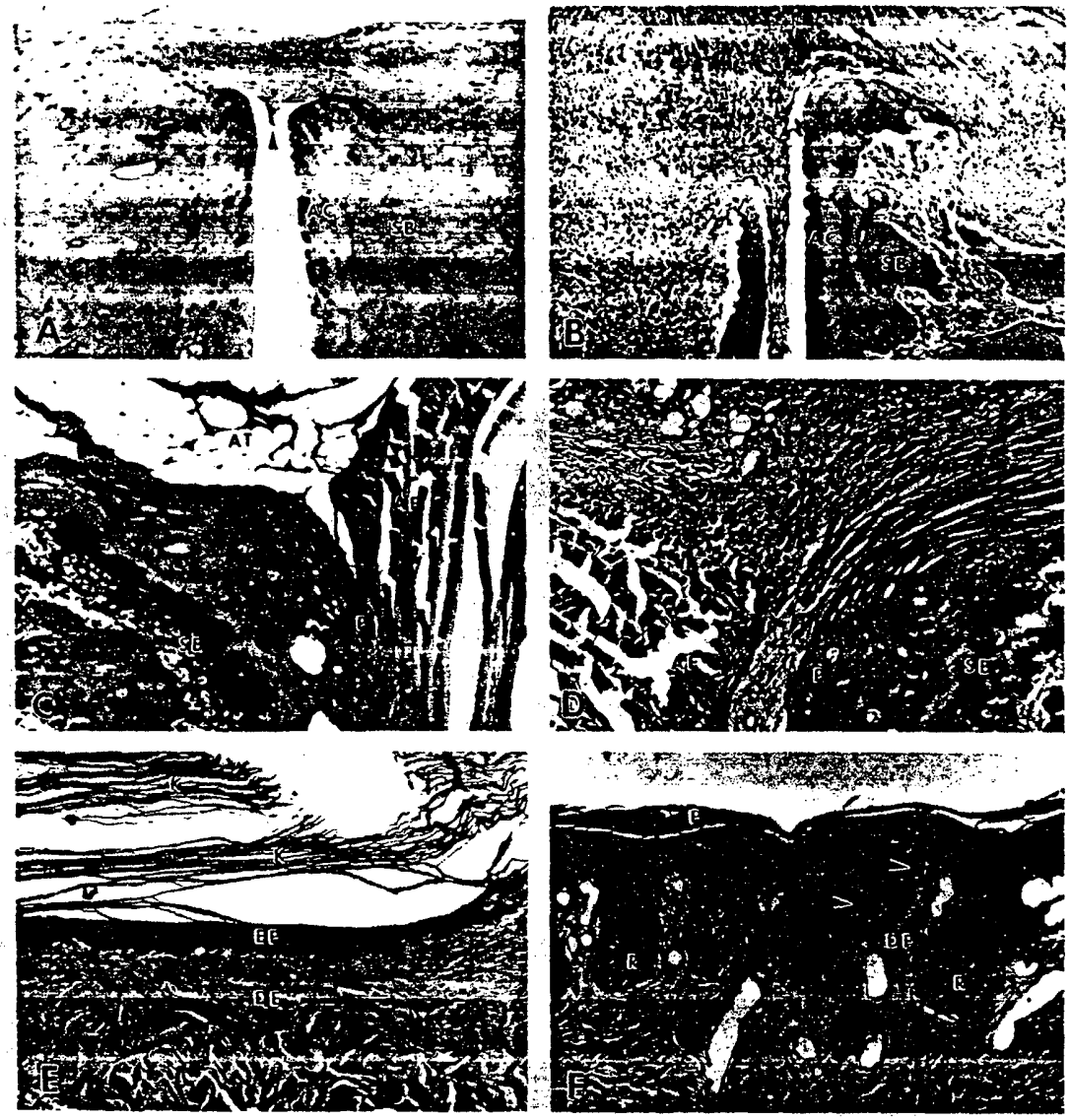

Figure 6. Peripheral and Axial Joint and Skin Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal tarsal joint. Synovium (arrowhead), articular cartilage (AC), subchondral bone (SB), and joint capsule (J) are labeled (78×).

(B) *Tarsal joint of a 4-month-old 21-4H male (same as in Figure 4D), showing chronic arthritis.* There is a marked inflammatory infiltrate in the joint capsule and synovium, with pannus (asterisks) eroding articular cartilage (AC) and subchondral bone (SB) on both sides of the joint (58.5×).

(C) *Normal tail intervertebral joint.* The annulus fibrosus (AF), vertebral end plate (P), ossification center of subchondral bone (SB), and periarticular adipose tissue (AT) are labeled (65×).

(D) *Tail intervertebral joint of a 4-month-old 21-4H male (same as in Figure 4D), oriented as a mirror image of (C), showing expansion of the periarticu-*lar connective tissue by mononuclear inflammation and fibrosis (asterisks), invading and disrupting the attachment of the outer layers of the annulus to the vertebral end plate (arrowheads). Annulus fibrosus, vertebral endplate, and subchondral bone are labeled as in (C) (58.5×).

(E) Normal tail skin. The keratin layer (K) overlies the epidermis (EP) and dermis (DE) (97.5×).

(F) Tail skin of a 3½-month-old 21-4 male (same as in Figure 5B), showing prominent, elongate, regular rete pegs (R) (psoriasiform epidermal hyperplasia), exocytosis of lymphocytes and neutrophils (arrowheads), parakeratosis (P), and dermal papillae (DP) containing inflammatory infiltrates (78×).

cial papillary dermis contained a diffuse infiltrate of neutrophils, lymphocytes, and plasma cells. Similar changes were seen in skin over the distal aspect of the digits.

Testis and Epididymis

Orchitis and epididymitis were prominent findings in the 21-4H males. The orchitis was manifested clinically by a progressive enlargement of the testes followed by testicular atrophy, with infertility ensuing by 3 months of age in most of the males. In contrast, the females showed little loss of fertility, even in the presence of persistent diarrhea. Histologically, the testicular tunica was thickened by connective tissue, which contained active angioblasts and fi-

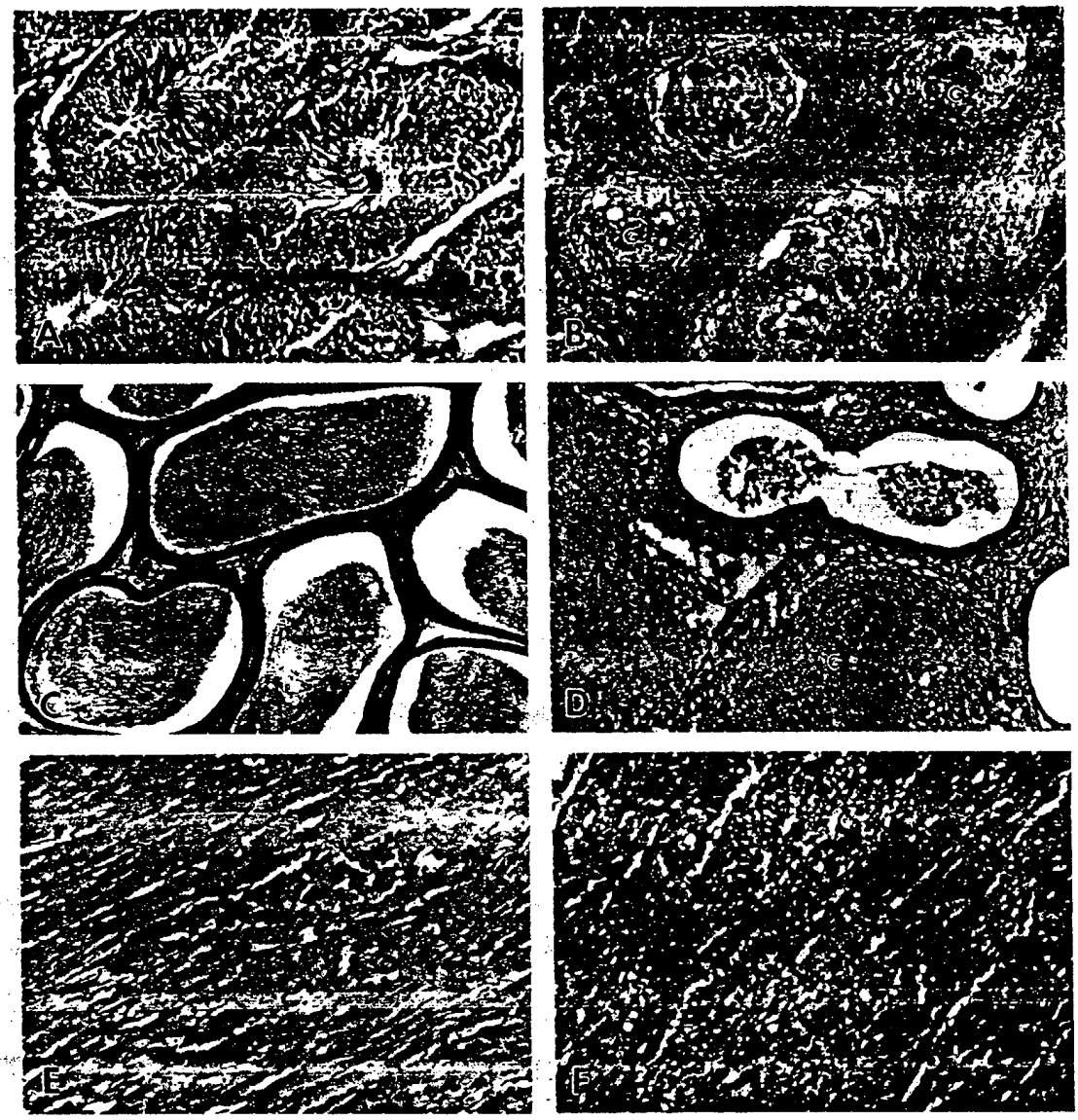

Figure 7. Male Genital Tract and Myocardial Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal testis (71.5×).

(B) Testis of a 3-month-old 21-4H male, showing chronic orchitis with an intense mononuclear cell interstitial inflammatory infiltrate and sperm granulomas (G) (71.5×).

(C) Normal epididymis (65×).

(D) Epididymis of a 3-month-old 21-4H male, showing chronic epididymitis with a granulomatous interstitial inflammatory cell infiltrate (I), sperm granuloma (G), and dilated tubules (T) containing degenerated inflammatory cells and no sperm (65×).

(E) Normal myocardium (78×).

(F) Myocardium of a 3-month-old 21-4H male (same as in Figure 3F), showing myocarditis with a prominent mononuclear inflammatory cell infiltrate separating the myofibers (78×).

broblasts as well as large numbers of lymphocytes and plasma cells. The testes often contained numerous granulomas with necrotic centers surrounded by epithelioid macrophages and giant cells and peripherally by lymphocytes, plasma cells, and fibrosis (Figure 7B). Central infarction of the testis was a common finding in the most severely affected specimens.

The epididymis frequently contained granulomas similar to those found in the testis, along with dilated tubules containing necrotic cellular debris. The interstitium of the epididymis was expanded by lymphocytes, plasma cells, epithelioid macrophages, and moderate fibrosis (Figure 7D).

Heart

Active inflammatory lesions were evident histologically in four of nine 21-4H hearts examined (Figure 7F). In one specimen, extensive multifocal lesions were seen, involving the ventricular walls and septum. The lesions con-

sisted of large numbers of lymphocytes and small numbers of plasma cells, macrophages, and eosinophils. The myofibers were widely separated by the inflammatory cells, and scattered karyorrhectic nuclei were seen. In the less severely affected specimens, infiltrates of lymphocytes and plasma cells were found at the root of the aortic valve. In more chronic lesions there was moderate fibrosis scattered throughout the myocardium accompanied by mild lymphocytic inflammation. In one animal the adventitia of the great vessels was infiltrated by large numbers of lymphocytes and plasma cells admixed with proliferating angioblasts and fibroblasts.

Eye and Central Nervous System

Mild keratitis and anterior uveitis were observed histologically in one of five eyes from 21-4H rats, one of five eyes from 21-4L rats, and none of four eyes from nontransgenic LEW rats. These findings were judged to be nonspecific, probably secondary to bacterial keratitis.

A peculiar neurologic syndrome was seen in all of the females and most of the males of the 21-4H line. This was manifested by cerebellar ataxia, with intermittent episodes of a stereotypical muscular dystonia, usually in response to handling or some other mild stimulus. Electrophysiologic studies during these episodes demonstrated increased muscular tone without evidence of a cortical seizure focus (data not shown). For several reasons, this abnormality was thought to result from a process distinct from that giving rise to the other lesions. Whereas the other lesions appeared after puberty and then progressed, the neurologic abnormality began within a few weeks after birth and showed no increase in severity thereafter. Unlike the other disease processes, the clinical pattern of the neurologic findings showed little variation from rat to rat. Furthermore, the histologic abnormalities associated with the neurologic disease, which involved primarily the spinal cord and cerebellum, were not inflammatory (data not shown). Finally, there was no evidence of neurologic disturbance in the transgenic F344 line that also showed diarrhea, nor in any of the other transgenic LEW lines.

Other Tissues

The following tissues were examined in at least one of the 21-4H rats showing diarrhea and found not to show histologic abnormalities: esophagus, lung, liver, kidney, adrenal, pancreas, penis, spleen, and thymus. Atrophy of thymus and spleen that was apparent to gross examination was a common finding, however, along with peripheral and mesenteric lymph node enlargement.

Clinical and Histologic Findings in Other Transgenic Lines

No clinical abnormalities were noted in any of the B27 transgenic LEW lines other than 21-4H. Histologic tissue surveys of several 21-4L rats revealed a mild degree of intestinal lymphoid hyperplasia and fibrosis as the only abnormality. Similar intestinal lesions were also found at a lower frequency in nontransgenic controls, and hence the significance of these findings in the 21-4L rats is not yet established. As noted above, almost all transgenic rats of the F344 line 33-3 showed diarrhea by 2 months of age.

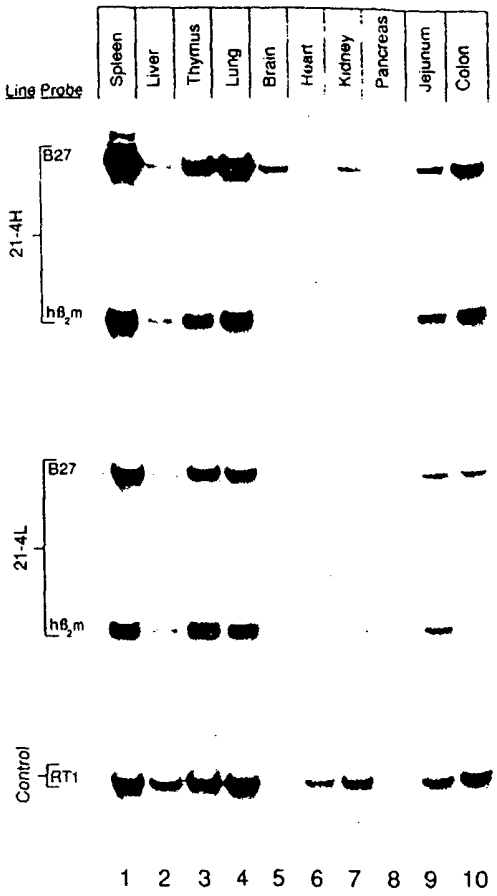

Figure 8. Northern Blot Analysis of HLA-B27, hβ2m, and RT1 mRNA: Tissue Survey

Total cellular RNA from tissues of 12-week-old male 21-4H, 21-4L, and nontransgenic control rats was subjected to denaturing agarose gel electrophoresis (10 μg per lane), transferred to nylon membranes, and hybridized to ^{32}P-labeled probes as described in Experimental Procedures and Figure 1. Membranes were exposed to XAR-5 film at −70°C with intensifying screens for 2–26 hr.

Tissue Distribution of mRNA Expression

Despite the striking differences in disease manifestations, the 21-4H and 21-4L lines showed similar cell surface expression of the transgene products in PBLs (Table 3; Figures 2A and 2B). It was thus of interest to compare the two lines with respect to the level and tissue distribution of mRNA transcripts of both transgenes. Northern blot analysis was carried out on total cellular RNA isolated from tissues of a limited number of rats of the 21-4H and 21-4L lines. HLA-B27 mRNA was detected with a 350 bp probe from the HLA-B 3′ untranslated region (probe C in Figure 1A), and hβ2m mRNA was detected with the same probe used to detect hβ2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp probe from the 3′ untranslated region of the RT1.A gene. Figures 8 and 9 contain results from age- and sex-matched representatives of the 21-4H and 21- 4L lines and a nontransgenic control.

As shown in Figure 8, the distribution and relative abundance of both B27 and hβ2m transgene transcripts among

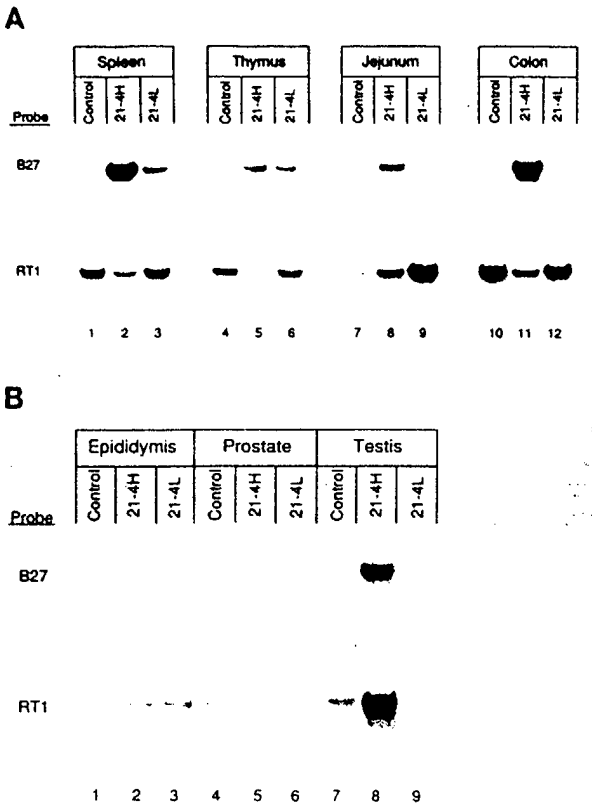

Figure 9. Northern Blot Analysis of HLA-B27 and RT1 mRNA: Comparative Analysis of Seven Tissues

(A) Tissue sources and methods were the same as described in Figure 8. Membranes were exposed 1–8 hr.

(B) Tissue sources were the same as described in Figure 8. Five micrograms of total cellular RNA was added per lane. Prostatic tissue in the 21-4H animal was difficult to identify because of severe atrophy, presumed to be due to loss of androgen stimulation. The membrane probed for B27 was exposed for 1 hr. A 10 hr exposure showed B27 transcripts in 21-4H epididymis (data not shown). The membrane probed for RT1 was exposed for 5 hr.

the various tissues examined were similar to those of the endogenous RT1 class I expression and typical of MHC class I gene expression (Klein, 1986). In addition, both transgenes produced mRNA transcripts of the predicted size.

Figure 9 shows direct comparisons of the 21-4H and 21-4L lines with respect to the relative amounts of B27 and RT1 transcripts in tissues affected by the disease process in the 21-4H line. The abundance of B27 transcripts was dramatically higher in the 21-4H rat than in the 21-4L rat in spleen, colon, and testis, and less markedly increased in jejunum and epididymis. In the thymus, the B27 transcripts were approximately equal in the two lines; however, this may have been a reflection of thymic atrophy in the 21-4H rats.

Although the apparent reduction of RT1 cell surface expression in PBLs was comparable in 21-4H and 21-4L rats (Figure 2C), at the level of mRNA there was no apparent reduction of RT1 transcripts in the 21-4L tissues examined. In contrast, the abundance of RT1 transcripts was markedly reduced in 21-4H spleen, thymus, and colon, compared with tissues from a nontransgenic rat. High expression of RT1 mRNA was found in the 21-4H testis and jejunum. In the case of testis, this probably reflects the intense infiltration of inflammatory cells seen histologically in this organ (Figure 7B), whereas an explanation for the finding in jejunum is less apparent.

Discussion

Integration and Expression of HLA-B27 and hβ$_2$m Transgenes in Rats

In an attempt to create an animal model of B27-associated disease, we developed transgenic technology in rats and produced inbred rats expressing both HLA-B27 and hβ$_2$m. Simultaneously, Mullins et al. (1990), using similar methods, were independently successful in producing transgenic rats expressing a mouse renin gene.

The levels of B27 and hβ$_2$m mRNA transcripts in the transgenic tissues paralleled those of the endogenous class I genes in nontransgenic tissues, suggesting that the transgenes were subject to physiologic regulation. It is interesting that the presence of the human transgenes resulted in an apparently reduced expression of the endogenous class I RT1 genes, at the level of cell surface protein expression in PBLs and/or at the level of mRNA, both in lymphoid and nonlymphoid tissue. The possibility was not excluded that the reduced binding of OX18 antibody to the transgenic PBLs was due to an effect of hβ$_2$m either on the number of cell surface RT1 class I molecules or on the affinity of the OX18 antibody for these molecules. However, such an effect would not explain the prominent reduction in RT1 mRNA transcripts seen in the 21-4H spleen, thymus, and colon.

Several transcriptional regulatory elements have been identified in the 200 bp 5′ to the transcription initiation site in murine class I MHC genes, including the binding site for the conserved nuclear factor KBF1 (David-Watine et al., 1990; Kieran et al., 1990), and homologous sequences are found in the HLA-B27 promoter region (Weiss et al., 1985). Thus, at least part of the inhibition of RT1 transcription in the 21-4H tissues might be explained by competition by the transgenes for nuclear factor binding.

The Inflammatory Disease of the 21-4H Transgenic Rats: Comparison with B27-Associated Disease in Humans

B27-associated disorders in humans encompass a spectrum of inflammatory diseases affecting predominantly the peripheral and axial musculoskeletal system, gastrointestinal tract, genital tract, integument, and eye (Table 1). Less common involvement of heart and nervous system and rare involvement of lung are also observed in these disorders (Bulkley and Roberts, 1973; Good, 1974; Taurog and Lipsky, 1990). The spontaneously arising disease in B27/hβ$_2$m transgenic rats showed a striking clinical and histologic similarity to B27-associated disease in humans, with inflammatory lesions of peripheral and axial joints, gut, male genital tract, nails, skin, and heart. The close resemblance of the findings in the transgenic rats

to B27-associated disease in humans strongly supports the conclusion that the B27 molecule itself participates in the pathogenesis of the various lesions found in different organ systems in the spondyloarthropathies.

The most prevalent site of inflammation in the transgenic rats appears to be the gastrointestinal tract. All of the 21-4H rats under observation for at least 6 months developed overt diarrhea, and a similar picture is emerging in the 33-3 line. These findings suggest that the events initiating the disease process occur in the gastrointestinal tract and that further investigation of the intestinal immunophysiology and immunopathology of the transgenic animals may provide some insight into the role of the B27 molecule in these events.

Numerous observations in humans support a causal link between factors in the gut and inflammatory joint disease. Peripheral and axial arthritis are common accompaniments of chronic inflammatory bowel disease even in the absence of B27 (Table 1), and recent evidence suggests that milder degrees of gastrointestinal inflammation are closely correlated with the occurrence of B27-associated joint disease in individuals without bowel symptoms. Histologic examination of endoscopically obtained biopsies in a large series of patients with reactive arthritis or ankylosing spondylitis indicated that over 60% had asymptomatic inflammatory lesions of the terminal ileum or colon (Cuvelier et al., 1987). Whether patients with B27-associated disease develop inflammatory lesions in the more proximal small intestine or stomach that might resemble those seen in the 21-4H rats is not known.

Although gastrointestinal inflammation in the transgenic rats was present equally in both sexes, arthritis occurred predominantly in males. This closely followed the pattern in humans, in whom males with ankylosing spondylitis, juvenile onset spondyloarthropathy, or reactive arthritis following genital infection outnumber females 3- to 10-fold. The prevalence of subclinical gastrointestinal inflammation in B27 individuals without rheumatic disease, either male or female, is not known. Both peripheral and axial arthritis occurred in the 21-4H rats. Clinically, the peripheral arthritis resembled that seen in other experimental models of arthritis in rats, such as those induced by complete Freund's adjuvant or streptococcal cell walls, with swelling and erythema of the proximal hind paw being the predominant lesion. Histologically, the involved joints showed lesions typical of experimental arthritis in rats, as well as B27-associated peripheral arthritis in humans, with synovial hyperplasia, inflammatory cell infiltration, pannus formation, and destruction of articular cartilage and bone (Greenwald and Diamond, 1988; Taurog et al., 1988b).

Axial arthritis, with inflammatory cell infiltration and periosteal reaction at the margins of the intervertebral discs, was seen histologically in the tails of 21-4H rats. This appears to be the same pathologic process that leads to the vertebral changes in ankylosing spondylitis, although histologic comparison of this lesion with human spondylitis is made difficult by the paucity of descriptions of early lesions in humans (Ball, 1971; Eulderink, 1990). More generally, the vertebral lesion in the 21-4H rats also closely resembles the enthesitis, inflammation at ligamentous attachments to bone, that is a pathologic hallmark of the B27-associated diseases in humans (Ball, 1971).

Dramatic psoriasiform skin and nail lesions developed in the 21-4H rats. These lesions show an extraordinary histologic resemblance to psoriatic lesions in humans. Although in most patients with psoriasis vulgaris there is no association with HLA-B27, lesions termed keratoderma blenorrhagica that are histologically indistinguishable from the psoriatic variant pustular psoriasis are commonly found in B27-associated reactive arthritis (Good, 1974; Keat, 1983). Furthermore, typical psoriasis vulgaris occasionally supervenes in patients initially presenting with reactive arthritis. Finally, a common pathogenetic mechanism between psoriasis vulgaris and B27-associated disease is suggested by the recent observation that both psoriasis vulgaris and the skin lesions of Reiter's syndrome appear to be significantly exacerbated in patients with coexistent infection with the human immunodeficiency virus HIV-1 (Duvic et al., 1987).

Another striking lesion in the 21-4H rats was orchitis, which was found in virtually all of the males, invariably in association with epididymitis. In humans, urogenital inflammation is prevalent in B27-associated diseases. Although urethritis in males with reactive arthritis is a common finding even in the absence of known urethral infection, prostatitis and epididymitis in males, cervicitis in females, and cystitis in both sexes have been described (Yli-Kerttula, 1984). Although there have been no reports of histologically confirmed orchitis associated with HLA-B27 or with B27-associated syndromes, clinical descriptions suggestive of orchitis have been published (Montanaro and Bennett, 1984). It is thus not altogether unlikely that the inflammatory process induced by B27 in the 21-4H rat testis has a milder human counterpart.

Inflammatory disease involving the root of the aortic valve and myocardium was found in the 21-4H rats. Both aortic insufficiency and cardiac conduction disturbances are well-documented complications of ankylosing spondylitis and reactive arthritis (Bergfeldt et al., 1988; Bulkley and Roberts, 1973; Good, 1974). Moreover, primary myocardial disease may also be relatively prevalent in ankylosing spondylitis (Brewerton et al., 1987). The cardiac pathology of the 21-4H rats, like the lesions in the peripheral and axial joints, gastrointestinal tract, skin, and male genital tract, thus appears to be a direct counterpart of a pathologic process in B27-associated human disease.

In comparing the pathologic lesions identified in the B27 transgenic rats with B27-associated disease in humans, only the neurologic disease in the 21-4H LEW line seemed to represent a significant anomaly. Occasional cases of either central or peripheral neurologic disease have been reported in association with B27-associated reactive arthritis (Good, 1974; Montanaro and Bennett, 1984; Taurog and Moore, 1986), but none of these has been characterized histologically, nor do their clinical descriptions resemble the findings in the 21-4H rats. As mentioned under Results, the neurologic lesions in the 21-4H rats appear to be temporally and histologically unrelated to the inflammatory disease seen in other organs.

Although the possibility cannot be excluded that the neurologic disturbance contributed indirectly to the inflammatory lesions, for example by disruption of the normal innervation of lymphoid tissue or gut (Anderson, 1990), the absence of neurologic disease in the 33-3 line, a second transgenic line exhibiting spontaneously occurring B27-associated disease, suggests that the neurologic disease in the 21-4H line is not a necessary part of the inflammatory process in other organ systems, but likely a result of a dominant insertional mutation. A complete description of the neurologic findings in the 21-4H line is in progress.

The Inflammatory Disease of B27/hβ_2m Transgenic Rats: Possible Mechanisms

It is unclear why overt inflammatory disease developed in only two of the seven transgenic rat lines, 21-4H and 33-3. It is unlikely that differences in postconceptional environment play a significant role in determining the phenotypes of the different transgenic lines, since segregation of the diseased phenotype with the 21-4H locus was uniformly observed in litters containing both 21-4H and 21-4L offspring. Insertional mutation appears unlikely as an explanation, since two independent transgenic lines developed aspects of a similar disease. Nor was evidence obtained for differences in B27 function, since the 21-4H and 21-4L lines comparably stimulated immune recognition of B27 by cytolytic T cells. The variation among transgenic rat lines most likely can be ascribed to either quantitative or qualitative differences in the expression of the transgenes or to differing effects of the transgene on the host genome.

The results presented in this study do not exclude the possibility that a human class I MHC gene other than HLA-B27 might also be capable of producing a disease process similar to that described here, nor do they exclude the possibility that the hβ_2m gene alone might be sufficient to produce disease. Studies are in progress to address these possibilities.

Several lines of evidence have suggested that interactions between B27 and bacterial products are involved in the pathogenesis of the spondyloarthropathies (Yu et al., 1989). Although the disease in the transgenic rats arose spontaneously in the apparent absence of infection by pathogens, the possibility must be considered that the pathogenesis involves interactions between B27 and commensal organisms such as the intestinal flora or pathogens not detected by routine serologic screening. Studies in which the transgenic rats are maintained germ free will be important in exploring this issue.

Despite extensive investigation of the structure and function of class I MHC genes in general and HLA-B27 in particular, it has so far not been possible to identify the molecular mechanism of the association of B27 with human disease. However, given the close resemblance of the spontaneous disease of the 21-4H line to B27-associated human disorders, a detailed cellular and molecular analysis of the B27/hβ_2m transgenic rats should enhance our understanding of the role of HLA-B27 in causing disease. It may also contribute to a broader understanding of the function of class I MHC molecules.

Experimental Procedures

Animals

Specific pathogen-free inbred Lewis/CrlBR (LEW) and Fischer F-344/CrlBR (F344) rats, and outbred Sprague-Dawley rats, were purchased from Charles River Laboratories, Boston, MA. Hybrid mice of the transgenic line 56-3 (Taurog et al., 1990), which express high levels of both B27 and hβ_2m on lymphoid cell surfaces, were bred in our animal colony. Animals were maintained in accordance with institutional guidelines.

Generation and Identification of Transgenic Rats

Immature LEW or F344 female rats were superovulated according to the method of Armstrong and Opavsky (1988) and bred with fertile males. The day following breeding, fertilized one-cell eggs were flushed from the oviduct of females exhibiting either vaginal plugs or sperm in vaginal lavage fluid. Eggs were held in Brinster's medium for 2 hr or less before microinjection. Microinjection of eggs and transfer to day 1 pseudopregnant Sprague-Dawley females were carried out essentially as described for mice (Brinster et al., 1985).

Two genomic clones were used for microinjection of fertilized rat eggs (Figure 1). The HLA-B27 gene encoding the HLA-B* 2705 subtype (Bodmer et al., 1990) was contained on a 6.5 kb EcoRI fragment (clone pE.1-B27; Taurog et al., 1988a; Taurog and El-Zaatari, 1988) and the hβ_2m gene was contained on a 15 kb SalI–PvuI fragment (clone pβ2m-13, the gift of Dr. H. L. Ploegh, Amsterdam, The Netherlands; Güssow et al., 1987). Each insert was separated from plasmid DNA by agarose gel electrophoresis and isolated by perchlorate elution (Chen and Thomas, 1980). The solution used for microinjection contained both fragments, each at 1.5 ng/μl.

Identification and quantitation of transgenes were determined in the founder animals and their progeny by dot-blot hybridization of genomic DNA isolated from tail biopsies, as previously described (Brinster et al., 1985). Genomic DNA was analyzed by hybridization with 5′ and 3′ flanking probes for the *HLA-B* locus, as previously described (probes A and B in Figure 1A; Taurog et al., 1988a), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the hβ_2m gene (probe D in Figure 1B).

RNA Analysis by Northern Blot Hybridization

Northern blot hybridization was carried out as described elsewhere (S. D. Maika, L. Laimonis, A. Messing, and R. E. Hammer, submitted). Briefly, total cellular RNA was extracted from tissues by the guanadinium isothiocyanate–CsCl procedure, separated on glyoxal agarose gels, and blotted onto nylon membranes. HLA-B27 mRNA was detected with the 350 bp HLA-B 3′ untranslated region probe pHLA-1.1 (probe C in Figure 1A; Koller et al., 1984), and hβ_2m mRNA was detected with the same 3.7 kb BglII fragment used to detect hβ_2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp PvuII–HindIII fragment containing the 3′ untranslated region of the RT1.A^a gene pBS3.3/1 (the gift of Dr. J. C. Howard, Cambridge, England; Rada et al., 1990). All stringency washes were carried out in 0.1× SSC, 0.5% SDS at 65°C.

Monoclonal Antibodies, Indirect Immunofluorescence, and Flow Cytometry

The following murine monoclonal antibodies were used: B.1.23.2, IgG_{2b}, binding a monomorphic determinant shared by HLA-B and -C molecules (Rebai and Malissen, 1983); BBM.1, IgG_{2b}, binding hβ_2m (Brodsky et al., 1979); and OX18, IgG_1, binding a monomorphic rat RT1 class I antigen (Fukumoto et al., 1982). P1.17, an IgG_{2a} myeloma, served as a negative control.

Indirect immunofluorescence was carried out as previously described (Taurog and El-Zaatari, 1988; Taurog et al., 1988a). Briefly, Ficoll–Hypaque-purified peripheral blood mononuclear cells were incubated with saturating concentrations of each monoclonal antibody, washed, then incubated with fluorescein-conjugated $F(ab)'_2$ fragments of goat anti-mouse Fcγ antibodies (Cappel Inc., Malvern, PA). After washing, the cells were fixed in 1% paraformaldehyde before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Viable lymphocytes were analyzed by gating of forward and 90° light scatter.

Generation and Analysis of Cytolytic T Cells

Primary alloimmunization by skin grafting was carried out by the method of Peter and Feldman (1972). Seven days after graft placement, recipient spleen cells were used as effector cells in a 4–6 hr ^{51}Cr release assay, as previously described (Taurog et al., 1988a). Two mouse L cell lines were used as target cells, one transfected with and expressing the hβ2m gene, the other transfected with and expressing both the HLA-B*2705 and hβ2m genes, as previously described (El-Zaatari et al., 1990).

Histology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Joints were embedded and sectioned following fixation and decalcification for 4–6 weeks in 10% disodium EDTA, as previously described (Taurog et al., 1988b), or following decalcification in 10% formic acid. Eyes were embedded in methacrylate before sectioning and staining.

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Expression of the DBA/2J *Ren-2* gene in the adrenal gland of transgenic mice

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To characterize further the tissue-specific control of the mouse *Ren-2* gene, and in particular its expression in the adrenal gland, we have introduced the DBA/2 *Ren-2* gene into the genome of *Ren-1*^{c}/*Ren-1*^{c} mice. Here we report our observations on *Ren-2* transgenic mice. Expression was found in the correct spectrum of tissues and included appropriate hormonal control in the submandibular gland. Quantitatively transcript levels varied both positively (adrenal gland and sex-accessory tissue) and negatively (submandibular gland and kidney) with respect to normal *Ren-2* expression. In the DBA/2 inbred mouse strain expression in the female adrenal gland cycles during oestrus between the X-zone and the zona fasciculata. Transgene expression within the adrenal gland was restricted to the X-zone. Therefore this phenotype, which is characteristic of most two-renin-gene strains of mice, contrasts with that found in the strain DBA/2 from which the transgene was derived. This suggests that cell-specific expression in the DBA/2 adrenal gland is mediated *in trans* by at least one additional locus. We demonstrate that suitable genetic crosses of the transgenic mice can partially restore the cycling phenotype.

Key words: adrenal/*in situ*/oestrus/renin/transgenic mice

Introduction

In mammals the primary source of circulating renin is the kidney where synthesis has been discretely localized to the juxtaglomerular cells (Hartcroft, 1963; Lacasse *et al.*, 1985). Recently, considerable attention has been given to extra-renal sites of renin expression, and there is evidence for renin synthesis in many organs, including the adrenal gland, testis, ovary and brain (Hirose *et al.*, 1980; Naruse *et al.*, 1984; Kim *et al.*, 1987). Although the significance of such expression is not yet understood, recent studies suggesting that angiotensin II promotes angiogenesis (Fernandez *et al.*, 1985b) and may be important for ovulation (Pellicer *et al.*, 1988) suggest that the renin–angiotensin system has physiological functions in addition to its classic role in the maintenance of blood pressure.

The mouse is unique with regards to extra-renal expression since the submandibular gland (SMG) can be the site of extremely high expression, both at the mRNA and protein level (Bing and Farup, 1965; Poulsen *et al.*, 1979). Those strains of mice that exhibit high SMG expression have been shown to carry a duplicated copy of the renin structural gene, *Ren-2* (Mullins *et al.*, 1982; Panthier *et al.*, 1982; Piccini *et al.*, 1982), which is closely linked to *Ren-1*^{d} (Abel and Gross, 1988). Strains that express low levels of SMG renin possess a single renin gene, *Ren-1*^{c}. Each of the three distinct mouse renin genes has a unique expression profile despite high nucleotide sequence conservation (Field *et al.*, 1984a; Holm *et al.*, 1984; Burt *et al.*, 1985). It is therefore not possible to extrapolate a rank order of expression for these genes from one tissue to another. The gene duplication event and juxtaposition of the two renin genes resulted in a modified genomic environment which may account for the strikingly different expression patterns of these highly homologous genes. In the SMG, *Ren-2* expression is ~100-fold higher than *Ren-1*^{c}, whilst to date there are no published reports of *Ren-1*^{d} transcripts having been detected in this tissue. This contrasts with the kidney, where all three transcripts are found at approximately equal levels (Field and Gross, 1985). Studies in the adrenal gland show that there are developmental differences in expression in addition to the variation found in adult animals, where *Ren-1*^{d} and *Ren-2* are equally abundant and *Ren-1*^{c} is not detectable (Field *et al.*, 1984b). Two additional tissues studied with respect to their patterns of renin gene expression are the testis and the male sex accessory tissue. Transcripts for all three genes have been identified in the testis with *Ren-1*^{d} being the most abundant, whilst the sex accessory tissue exhibits a very high level of *Ren-1*^{c} transcripts but little, if any, *Ren-1*^{d} or *Ren-2* derived transcripts (Fabian *et al.*, 1989). The relative expression of these genes in different tissues is summarized in Table I, which takes into account the number of renin-expressing cells in each tissue.

A striking fluctuation in the cell specificity of adrenal renin expression during oestrus has been observed by McGowan (1987). In the adult female mouse three phenotypes of adrenal renin expression are found. Firstly, a distinction can be made between strains with the *Ren-1*^{c} gene, in which expression is not detectable, and those carrying the two renin

Table I. Expression of mouse renin genes of various tissues

	Ren-1^c	*Ren-1*^{d}	*Ren-2*
Testis	+	+ +	+
SMG	+	–	+ + +[a]
Sex-accessory gland	+ +	–	–
Adrenal gland			
Zona fasciculata	–	+[b,c]	+[b,c]
X-zone	–	+[b]	+[b]
Kidney	+ + +	+ + +	+ + +

[a]Males express significantly higher than females.
[b]Cycling of expression between the zones occurs in DBA substrains.
[c]Females express significantly higher than males.

genes *Ren-1*^{d} and *Ren-2*, which are both expressed throughout oestrus. Secondly, within the two-renin-gene strains, two patterns of cell specificity are found. The simplest phenotype is constitutive expression within the X-zone of the adrenal cortex. A more complicated phenotype is observed in the closely related strains, DBA/1 and DBA/2. Whilst the relative abundance of *Ren-1*^{d} and *Ren-2* transcripts does not alter, the site of expression within the adrenal cortex changes in a cyclic manner during oestrus. At pro-oestrus, renin transcripts are present in approximately equal abundance in both the X-zone and the zona fasciculata. During oestrus, expression is only found in the zona fasciculata and a dramatic change in cell specificity occurs in the transition from oestrus to met-oestrus. At this time renin transcripts in the zona fasciculata decrease to an undetectable level, and are replaced by expression localized exclusively in the X-zone. The fourth stage, di-oestrus, completes the cycle and is characterized by renin expression in both the X-zone and the zona fasciculata with the latter having a significantly higher abundance of transcripts. Such observations further demonstrate the complexity of renin gene expression in the mouse and it is of considerable interest to define what aspects of differential structure act in *cis* to produce the differences described above. Transgenic mice provide a means of addressing these questions and also permit the study of gene expression during ontogeny and in varying physiological states. As a starting point for such analyses of the mouse renin genes we have constructed mice possessing both *Ren-1*^{c} and *Ren-2*^{d} by introducing the entire DBA/2 *Ren-2* gene into the germline of mice which carry only the *Ren-1* locus (*Ren-1*^{c}). Here we describe our observations on the expression of this transgene, and the effect of oestrus on the cell specificity of the transgene expression within the adrenal gland.

Results

Isolation of transgenic animals

To construct transgenic mice carrying the *Ren-2* gene, a 24 kb *Xho*I restriction fragment was isolated from a *Ren-2* cosmid clone isolated in this laboratory (Field *et al.*, 1984b). This fragment contains 5.3 kb 5′ of the major transcription start site and 9.5 kb 3′ of the polyadenylation site. The DNA was prepared as described and injected into the male pronucleus of BCF_2 eggs. Ninety-nine mice were born of which 90 survived to weaning, and Southern blot analysis was performed on DNA isolated from tail biopsies of each animal. Restriction digests were carried out with the enzyme *Pvu*II and blots were probed with an exon I–III specific 300 bp *Pst*I–*Kpn*I restriction fragment, isolated from the mouse renin cDNA clone, pDD-1D2 (Field *et al.*, 1984b).

Figure 1(A) shows a Southern blot identifying the presence of *Ren-2*-specific 8.5 and 0.8 kb *Pvu*II fragments, in three of the mice tested. The additional 3 and 0.6 kb fragments are derived from the endogenous *Ren-1*^{c} gene. In total, 19 transgenic mice were identified of which 14 were successfully bred to yield positive offspring. The structure of the *Ren-2* gene is indicated in Figure 1(B), which indicates the relevant *Pvu*II sites that distinguish the transgene from the endogenous *Ren-1*^{c} gene.

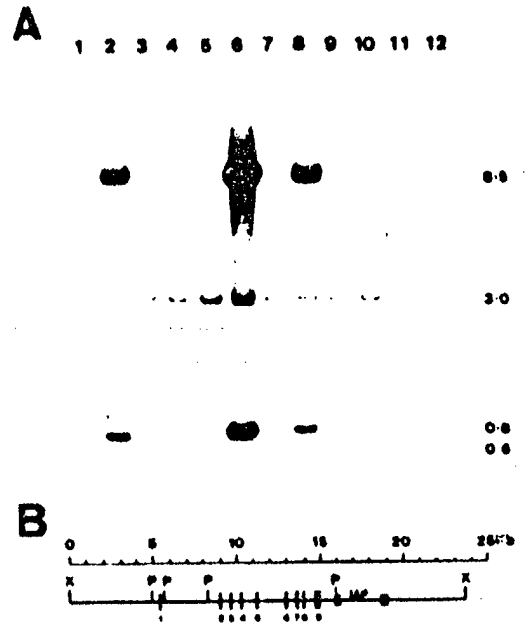

Fig. 1. Southern blot analysis of progeny from microinjection. (A) *Pvu*II-digested DNA samples (10 μg) from each animal were electrophoresed on a 0.8% agarose gel, Southern blotted, and probed with a 300 bp renin cDNA probe specific to the 5′ end of the gene (exons I–III). The probe was labelled with [^{32}P]α-dCTP to a specific activity of 1×10^9 d.p.m./μg. lanes 1–11, tail DNA samples; lane 12, DBA/2. The sizes of the restriction fragments (Kb) are shown at the right of the figure. (B) Structure of the DBA/2 *Ren-2* gene. The positions of the nine exons of the *Ren-2* gene are indicated within the 24 kb *Xho*I (X) restriction fragment used in these studies. The location of the intra-cisternal A particle (IAP) element is indicated 3′ of the gene, and diagnostic *Pvu*II sites are denoted by the letter P.

Expression of the *Xho*I transgene

Prior to the analysis of transgene expression within the adrenal gland, we determined whether the *Ren-2* transgene was expressed in a correct tissue-specific manner. Males from two-renin-gene strains (such as DBA/2) characteristically express *Ren-2* at a high level in the SMG, in which expression has been localized to the granular convoluted tubule (GCT) cells (Hirose *et al.*, 1983; Lacasse *et al.*, 1985). The transgenic founder animals were therefore screened for the ability to express *Ren-2* in this tissue, and seven mice were found to express the transgene in a cell-specific manner, at between 5 and 10% of the level in the SMG of a DBA/2 mouse. Figure 2 (left panel) shows *in situ* hybridization analysis of the SMG from male founder TgX2 with the renin antisense probe pSLM and the non-hybridizing sense probe pMLS. While pMLS shows no specific hybridization, pSLM clearly demonstrates a strong signal specific to the GCT cells. In order to determine if the sexual dimorphism characteristic of *Ren-2* (and *Ren-1*^{c}) expression in the SMG is also observed for the transgene, SMG RNA from positive male and female progeny of TgX2 were screened with a gene-specific primer extension assay (Field and Gross, 1985) and the results are shown in Figure 2 (right panel). Expression of the transgene was found to be significantly higher in the male SMG (lane 3), than in the female SMG (lane 4) which in this exposure is undetectable.

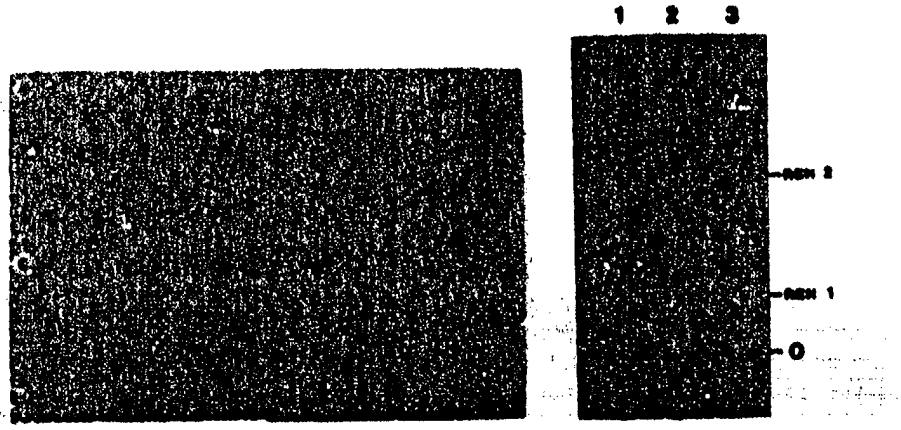

Fig. 2. (Left panel) *In situ* hybridization of TgX2 SMG. Frozen sections (8 μm) of SMG tissue from TgX2 were hybridized to a ^{35}S-labelled renin cRNA probe (pSLM) and its complement (pMLS), and exposed for 17 days. (a,b) TgX2 SMG hybridized to pSLM, bright field and dark field. (c,d) TgX2 SMG hybridized to pMLS, bright field and dark field. (Right panel) Effect of androgen on SMG transgene expression. Primer extension analysis of SMG RNA (20 μg) from positive male (lane 2) and female (lane 3) progeny of TgX2. Lane 1, DBA/2 kidney (80 μg). *Ren-1* and *Ren-2* specific extension products are indicated, and excess oligonucleotide primer is shown by 'O'.

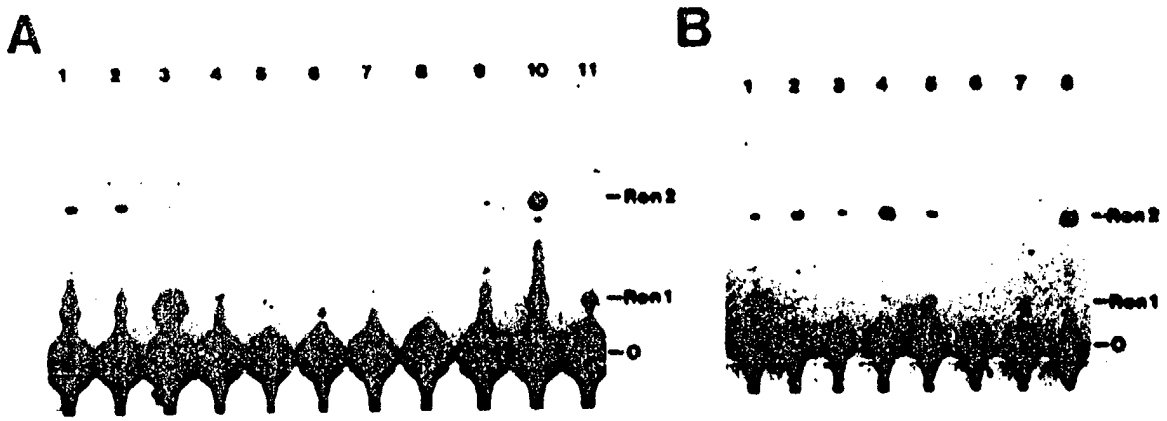

Fig. 3. (A) Tissue survey of male TgX2 progeny. Primer extension analysis of RNA prepared from the following tissues: lane 1, kidney; lane 2, testes; lane 3, sex accessory tissue; lane 4, liver; lane 5, heart; lane 6, lung; lane 7, spleen; lane 8, muscle; lane 9, brain; lane 10, male DBA/2 kidney; lane 11, male BCF_1 SMG. Excess of oligonucleotide (38mer) is indicated by 'O', and the extension products observed for *Ren-1* and *Ren-2* are indicated. For each experiment, 100 μg of RNA was used except for: SMG (20 μg), heart (71 μg), lung (65 μg) and muscle (45 μg). (B) Transgene expression in the adrenal gland. Primer extension analysis of RNA prepared from adrenal glands of transgenic and control mice. Lane 1, TgX5 female; lane 2, TgX11 female; lane 3, TgX2 female; lane 4, TgX6 female; lane 5, DBA/2Ha female; lane 6, C57BL/10 male; lane 7, DBA/2 kidney (male) 80 μg; lane 8, DBA/2Ha SMG (male) 0.8 μg. All adrenal samples used 10 μg of RNA except lane 2 (TgX11) in which 5 μg was used. *Ren-1* and *Ren-2* extension products are indicated. Oligonucleotide primer is shown by 'O'.

Upon testosterone induction of females carrying the transgene, SMG expression of *Ren-2* increases to approximately the same levels as that observed in males of the same line (data not shown). Taken together, these results demonstrate that within the SMG the transgene is discretely expressed and responds in a manner similar to that of the wild-type *Ren-2* gene upon androgen-induced differentiation.

To characterize further the tissue specificity of transgene expression, total RNA was prepared from a wide range of tissues from a mature transgene-positive male and screened by primer extension to determine whether *Ren-2* transcripts were present (Figure 3A and B). Figure 3(A) shows that expression of *Ren-2* is observed in the kidney and testis, both sites in which the wild-type *Ren-2* gene is known to be transcribed. In addition, expression was detectable in the brain and sex-accessory tissue. In contrast to all other expressing tissues, Ren-2 transcripts within the brain were found to be of abnormal length and their relevance is at present unclear (see Discussion). The level of expression within the sex-accessory tissue, although low, was greater than that found in a DBA/2 mouse (Fabian *et al.*, 1989) and reflects a site of enhanced transgene expression. The ability of this tissue to express *Ren-2*-derived transgenes has been independently confirmed using fusion constructs (this laboratory, in preparation). Expression was not evident in liver, heart, lung, spleen or skeletal muscle, a finding consistent with the transgene being regulated correctly. Having confirmed that the transgene exhibited appropriate

Fig. 4.

Fig. 5.

tissue- and cell-specific expression, we focused on the primary tissue of interest in the present study, the female adrenal gland. RNA from the adrenal glands of female mice from each transgenic line was assayed for *Ren-2* transcripts and Figure 3(B) indicates the expression observed in adrenal tissue of females from lines TgX2, TgX5, TgX6 and TgX11. When compared to the expression of the wild type DBA/2 *Ren-2* gene (Lane 5), it can be seen that the transgene is expressed at an equivalent (TgX2, TgX5) or significantly greater level (TgX6, TgX11).

Localization of Ren-2 expression within the adrenal gland

While *Ren-1*^{d} and *Ren-2* are equivalently expressed in the adult female adrenal gland at all stages of oestrus (this laboratory, unpublished), *Ren-1*^{c} is not expressed. It was therefore possible to use *in situ* hybridization to localize expression of the transgene within this tissue. It was of interest to determine firstly whether the high levels of expression observed were due to constitutive expression throughout the gland or to expression in a discrete subset of cells, and, secondly, whether expression varied qualitatively or quantitatively during the oestrus cycle. Adrenal glands were removed at each stage of oestrus from transgenic mice (lines TgX2 and TgX6) and BCF_2 control animals. After sectioning the tissues were hybridized to a renin-specific ^{35}S-labelled cRNA probe (Figure 4). Control animals did not express renin transcripts at any stage of oestrus (Figure 4 i–p). In contrast, TgX2-derived females (and also TgX6-derived females, data not shown) expressed the transgene constitutively in the X-zone. No quantitative or qualitative variation was seen at any of the four stages of oestrus (Figure 4 a–h). Lipid vacuoles, which are a common but variable feature of the female adrenal cortex, are evident in many sections but their appearance did not correlate with either oestrus or renin expression.

Induction of oestrus-dependent transgene expression

A primary difference between the TgX2 transgenic animals and DBA/2 is the genetic background in which the *Ren-2* gene resides. Therefore given the constitutive expression of the transgene in the adrenal X-zone, we postulated that an additional locus or loci may be involved in controlling oestrus-dependent cycling of expression. In support of this, female mice (BXD) derived from an F1 cross between C57BL/6J (*Ren-1*^{c}) and DBA/2 (*Ren-1*^{d}, *Ren-2*) exhibited a modified cycling phenotype in which renin mRNA concentrations in the zona fasciculata rose and fell but were never undetectable, and expression in the X-zone was constitutive (Figure 5, left panel a–d). Moreover, this phenotype is in contrast to that of DBA/2 in which X-zone expression was absent during oestrus (Figure 5, left panel e–h).

Since F1 females carrying single copies of DBA/2-specific loci demonstrated the ability to vary expression with oestrus, we carried out a genetic cross between a transgenic female mouse and a D2C male. The D2C strain, (D2.C-Idh-1,Pep-3) carries the Balb/cAnPt renin locus on a DBA/2N background (Mock *et al.*, 1987) and does not express its single renin gene (*Ren-1*^{c}) in the adult adrenal gland. Since the DBA/2 and D2C strains are congenic, progeny derived from the above cross were essentially heterozygous for all DBA/2 loci.

Female progeny were tested for the presence of the transgene and positive animals were staged for oestrus. *In situ* hybridization analysis revealed that these mice had a modified phenotype similar to that of the BXD F1 animals (Figure 5, right panel). Renin mRNA was observed in the zona fasciculata and during progression from oestrus (i,j) through met-oestrus (k,l) to di-oestrus (m,n) this expression first diminished and then increased. Expression in the X-zone was significantly greater than that in the zona fasciculata, and remained high during met-oestrus, further demonstrating the similarity to the phenotype observed in BXD F1 females.

A single copy of one or more DBA/2-specific loci is therefore sufficient, and essential, to induce partial cycling of transgene expression in the zona fasciculata but is insufficient to induce cycling in the X-zone. It is possible that complete quantitative restoration of the cycling phenotype may be achieved by introduction of a second copy of the DBA/2 locus (or loci) responsible and breeding experiments to test this are presently being carried out.

Discussion

The aim of the present study was to analyse the tissue-specific regulation of the mouse *Ren-2* gene in the adrenal gland by introducing a cloned copy of this gene onto a *Ren-1*^{c}/*Ren-1*^{c} genetic background. The tissue specificity of transgene expression was determined by primer extension and *in situ* hybridization, and a detailed study of adrenal expression performed.

Our analysis of transgenic mice carrying the DBA/2 *Ren-2* gene with 5.3 kb of 5′ and 9.5 kb of 3′ flanking sequence respectively demonstrates that although such a construct will direct significant expression within the anticipated spectrum of tissues, the absolute level of expression is not equivalent

Fig. 4. *In situ* hybridization analysis of female TgX2 adrenal glands. Adrenal glands were removed from staged female BCF_2 and transgenic line TgX2-derived female mice. Frozen sections (8 μm) were hybridized with the [^{35}S]renin cRNA probe pSLM. (a,b) TgX2 pro-oestrus; bright field, dark field. (c,d) TgX2 oestrus; bright field, dark field. (e,f) TgX2 met-oestrus; bright field, dark field. (g,h) TgX2 di-oestrus; bright field, dark field. (i,j) BCF_2 pro-oestrus; bright field, dark field. (k,l) BCF_2 oestrus; bright field, dark field. (m,n) BCF_2 met-oestrus; bright field, dark field. (o,p) BCF_2 di-oestrus; bright field, dark field. All sections were exposed for 8 days except those for met-oestrus which were exposed for 15 days.

Fig. 5. (Left panel) Expression of renin mRNA in the adrenal gland of female DBA/2 × C57BL/6J mice (BXD F1). (a,b) BXD F1 pro-oestrus; bright field, dark field. (c,d) BXD F1 oestrus; bright field, dark field. (e,f) DBA/2 pro-oestrus; bright field, dark field. (g,h) DBA/2 oestrus; bright field, dark field. Hybridized sections were exposed for 19 days except for (e) and (f) which were exposed for 15 days. (Right panel) Induction of transgene expression in the zona fasciculata. Frozen sections (8 μm) were prepared from the adrenal glands of staged female transgene-positive progeny from a cross between a male D2C mouse and a female from the TgX2 transgenic line. Sections were hybridized to ^{35}S-labelled transcripts synthesized from pSLM, and exposed to emulsion for 13 days. (i,j) oestrus; bright field, dark field. (k,l) met-oestrus; bright field, dark field. (m,n) di-oestrus, bright field, dark field.

to the endogenous *Ren-2* gene in a DBA/2 mouse. Transcript levels in the SMG and kidney were ~10% of the level found in DBA/2 but in the adrenal gland, transgene-derived transcripts were found to be extremely abundant, exceeding the level of *Ren 2* expression in the female DBA/2 mouse. The adrenal glands of male mice did not express significant amounts of *Ren-2* although, as for DBA/2, low levels were detectable throughout the zona fasciculata. The lack of X-zone expression in male mice possessing *Ren-1*^{c} and *Ren-2* is due to the degeneration of this zone in the presence of high circulating levels of androgen, which are also responsible for difference in SMG renin levels between male and female mice. This difference in SMG expression is reflected in the transgenic animals, and demonstrates that the transgene is able to respond normally to the presence of androgens. In one tissue, the brain, transcripts were found by Northern blotting to be greater than the expected length. The origin of these transcripts is presently unknown, but may be due to abnormal splicing or the use of a cryptic promoter or termination site. The relevance of these transcripts is unclear. Further characterization by *in situ* hybridization analysis and RNase protection assays will clarify their origin.

The site of integration can dramatically affect transgene expression, which often does not correlate with copy number (Palmiter and Brinster, 1986). This may explain why the expression of the *Ren-2* transgene did not reach wild-type levels in the SMG and kidney. Several lines showed high levels of adrenal gland renin expression suggesting that the site of integration may be optimal for expression in some tissues but not others. Position-dependent expression may be circumvented by the construction of a minilocus (Grosvel *et al.*, 1987) which permits copy number-dependent expression irrespective of the insertion site.

Tronik *et al.* (1987) recently introduced a similar construct into the mouse germline and observed variable but higher levels of expression in the SMG and kidney than we report here. However, as in this study, expression was not proportional to copy number. Since the shorter transgene used in their study was isolated from the SWR inbred strain and introduced into mice of a different genetic background, this precludes a direct comparison. However, since several tissue-specific enhancers may be present within a single gene and direct different expression patterns (Hammer *et al.*, 1987), it is possible that the two constructs do not possess equivalent control elements.

Transgene expression in the adrenal gland

Renin transcripts in female DBA/2 adrenal glands exhibited cycling between the zona fasciculata and the X-zone, as described previously. *Ren-2* transcripts in stage-matched transgenic animals were found exclusively in the X-zone, a feature characteristic of all two-renin-gene strains tested with the exception of DBA/1 and DBA/2 (McGowan, 1987). Thus, expression of the *Ren-2* transgene produces an exact phenocopy of all two gene strains except the one from which the gene was isolated (DBA/2). This strongly supports the evidence established for a *Ren*-independent, DBA-specific locus being involved in the adrenal gland phenotype described.

These results provide evidence that a *cis*-acting element capable of promoting expression within the X-zone of the mouse adrenal gland is present within or very close (between 5.3 kb upstream and 9.5 kb downstream) to the DBA/2 *Ren-2* gene. On the genetic background provided by the transgenic animals (C57BL/10Ros-p^d × C3Hf/HeRos) no expression was found in the zona fasciculata at any stage in oestrus. However, when the transgene was introduced onto the heterozygous DBA/2 background, expression was found in the zona fasciculata in addition to the X-zone. Therefore, expression in the zona fasciculata requires the combination of a *cis*-acting element provided by the transgene and a DBA-specific *trans*-acting factor. Whether the *cis*-acting elements responsible for expression in the zona fasciculata and the X-zone are identical or distinct entities cannot be determined from the present study.

The mechanism and significance of the cycling event

We intend to determine, genetically, the number of loci involved in the cycling of adrenal renin expression, and the mechanism by which it is mediated. The mechanism underlying this phenotype may involve transcriptional control and/or variation in mRNA stability. One of the possible models to explain why zona fasciculata expression and the cycling event arise simultaneously, would be that a DBA-specific positive regulatory factor is synthesized or reversibly activated in a cyclical manner during oestrus, and assumes that renin mRNA half-life is extremely short.

Control at the level of mRNA stability would seem probable since the changes in renin mRNA concentrations are very dramatic, particularly at met-oestrus, which in the mouse lasts only a few hours. For such changes to be solely controlled at the transcriptional level would require not only very tight regulation of transcription but also that renin mRNA half-life be extremely short. Although mRNA stability is a documented mechanism of gene control (Brawerman, 1987; Raghow, 1987), there has been no indication to date that it is involved in controlling renin gene expression. Recent evidence from *in situ* hybridization experiments using [^{3}H]poly(UTP) as a probe shows that there is no detectable variation in the abundance of polyadenylated RNA within the adrenal gland of DBA/2 mice during oestrus. A similar analysis of 21-hydroxylase mRNA distribution (using a probe kindly supplied by J.Seidman) has shown that transcripts are constitutively present in both the X-zone and the zona fasciculata of DBA/2 mice. Together, these experiments indicate that oestrus-dependent cycling of transcript abundance is renin specific and not due to the cyclic variation in cellular mRNA content. Additional transgenic mice carrying marker genes fused to renin 5' sequences will directly assess transcription regulation of the *Ren-2* promoter and clarify the role of mRNA stability.

Ovarian renin levels have also been shown to change during oestrus (Ferrandez *et al.*, 1985a; Sealey *et al.*, 1987) and suppression of angiotensin II, the active product of the renin–angiotensin cascade, has been suggested to lead to inhibition of ovulation (Pellicer *et al.*, 1988). Other genes have been observed to vary their expression during oestrus (Ivell *et al.*, 1985; Jin *et al.*, 1988; Van Tol *et al.*, 1988), but these studies do not suggest that the cell specificity of expression is altered as is the case in the present study. The function of the oestrus-dependent cycling event is at present unknown. The adrenal renin–angiotensin system has been demonstrated to play a role in potassium-stimulated aldosterone production, and angiotensin II is known to elicit a mitogenic response in bovine adrenocortical cells (Gill *et al.*, 1977; Nakamura *et al.*, 1985). Further, angiotensin

II has been implicated in angiogenesis, an important element of the oestrus cycle (Fernandez *et al.*, 1985b). Given the timescale of the cycling events, it is difficult to envisage these latter functions in this instance. However, since certain wild mouse species also express adrenal renin in a cyclic manner this phenotype may well have a hitherto unknown physiological function, possibly in regulation of steroid or catecholamine synthesis.

Materials and methods

Preparation of DNA for microinjection

DNA was prepared by digestion of the cosmid clone cosDBA-1 (Field *et al.*, 1984b) with *Xho*I, and subsequent isolation of the 24 kb *Xho*I fragment containing the *Ren-2* gene on a 10–20% sucrose gradient in 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 200 mM sodium acetate. Fractions containing the required fragment were pooled and recovered by ethanol precipitation before being centrifuged on a CsCl gradient as described by Hogan *et al.* (1986).

The DNA preparation was diluted to a final concentration of 1 μg/ml in injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), and stored in aliquots at −20°C prior to use.

Southern blotting

Southern blots were prepared by the method of Southern (1975). Hybridization probes were prepared using the random hexanucleotide priming method of Feinberg and Vogelstein (1983), and hybridizations were carried out according to Jeffreys *et al.* (1980). Final washes were in 1 × SSC, 0.1% SDS at 65°C, and filters were exposed to Kodak XAR-5 film with an intensification screen at −70°C.

In situ hybridization

Tissues were snap-frozen in 2-methyl butane on dry ice–ethanol and stored under liquid nitrogen prior to use. Frozen sections (8 μm) were cut using a Reichert Histostat and hybridized with ^{35}S-labelled cRNA probes of 50–150 nucleotides in length prepared by partial hydrolysis of pSLM or pMLS derived transcripts as described by McGowan (1987). These plasmids contain a 911 bp *Sac*I–*Pst*I mouse SMG renin cDNA fragment isolated from pDD-ID2 (Field *et al.*, 1984) cloned into the vectors pSP64 (pMLS) and pSP65 (pSLM). For the adrenal gland studies, slides of DBA/2 SMG and kidney were included as controls. As a negative control each experiment included hybridizations with a ^{35}S-labelled renin RNA probe (pMLS transcript).

Primer extension assays

Primer extension reactions were carried out on total RNA preparations, essentially as described by Field and Gross (1985) except that AMV reverse transcriptase was obtained from BRL. This assay uses a 38mer oligonucleotide (Field and Gross, 1985) which hybridizes equally well to both *Ren-2* and *Ren-1*^{d} transcripts. Upon reverse-transcription in the presence of ddCTP instead of dCTP, gene-specific products of 55 and 43 nucleotides are produced for *Ren-2* and *Ren-1*^{d} respectively. The 38mer oligonucleotide was 5′ end-labelled with [^{32}P-γ]ATP (7000 Ci/mmol, purchased from NEN) using T_4 polynucleotide kinase (Maniatis *et al.*, 1982). Extension products were electrophoresed on 8% acrylamide urea gels and placed on Kodak XAR film at −70°C, with an intensifying screen.

RNA and DNA isolation

Total RNA was isolated from mature mice either by homogenization of tissues in an equal mixture of phenol:chloroform:isoamyl alcohol (25:24:1) and TLES (0.1 M Tris–HCl, pH 9.0, 0.1 M LiCl, 100 mM EDTA, 1% SDS), or by homogenization in guanidine isothiocyanate (Chirgwin *et al.*, 1979). In both procedures the RNA was purified by centrifugation of the aqueous extract through 5.7 M CsCl, 0.1 M EDTA, pH 7.5, as described by Mullins *et al.* (1982). Pellets of RNA were resuspended in DEPC-treated water and ethanol precipitated. After final resuspension in DEPC-treated water all RNAs were stored at −70°C.

DNA was prepared from small pieces of tail tissue according to the procedure of Hanahan (Hogan *et al.*, 1986). Large-scale genomic DNA preparations were made from liver tissue by the method of Jeffreys *et al.* (1980).

Construction of transgenic animals

Animals were maintained on a 12 h light–dark cycle. Fertilized eggs (BCF_2) were derived from a cross of C57BL/10Ros-b^d × C3H/HeRos F1 mice after superovulation of immature females (3–5 weeks of age) by injection of 10 units of pregnant mare's serum gonadotropin (Diosynth Inc., Chicago, IL) 60 h prior to the midpoint of the dark cycle in which mating occurred, and 10 units of human chorionic gonadotrophin (Sigma) 12 h prior to the midpoint. Eggs were cultured, microinjected and reimplanted as described by Hogan *et al.* (1986). To maximize survival rate, eggs were fostered by Ha/ICR females that had already successfully weaned a litter. At weaning the mice were tested for the presence of the transgene by Southern blotting of tail DNA preparations. All mice used in this study were obtained from the West Seneca L. laboratories (West Seneca, NY), with the exception of the D2.C-Idh-1, Pep3 (D2C) mice (a DBA/2N congenic strain carrying the BALB/cAnN renin locus) which were kindly provided by Dr M. Potter (NIH).

Analysis of oestrus

Healthy young female mice (6–12 weeks old) were housed in a room containing cages of breeding males, in order to stimulate a regular oestrus cycle. Animals were maintained on a 12 h light/dark schedule and allowed 1–2 weeks to adjust to new surroundings before vaginal smearing commenced. The smears were performed mid-morning on a daily basis, cells being dried on microscope slides and then stained with methylene blue. The stage of oestrus was then determined by viewing the cell population present as described by Allen (1922). The mice were followed for at least one full cycle of oestrus before adrenal glands were removed. Those that were not cycling consistently were not used in the study, and mice sharing a cage were killed at the same time, since irregularities in the cycles were observed after separation or if a dominant female was removed. After killing the animals, the uterus and ovary were examined to confirm correct staging.

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HLA-B27 IN INBRED AND NON-INBRED TRANSGENIC MICE

Cell Surface Expression and Recognition as an Alloantigen in the Absence of Human β_2-Microglobulin[1]

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A gene encoding the H chain of the human class I MHC Ag HLA-B27 was introduced into the germ lines of inbred C57BL/6 (B6) and non-inbred (B6 × SJL/J) F_2 mice. By immunofluorescence and flow cytometry, the HLA-B27 gene product was expressed on lymphoid cells at levels comparable to the endogenous H-2^b and H-2^s class I MHC molecules. In both primary and secondary MLC between responder spleen cells from non-transgenic (B6 × SJL/J) F_1 mice and transgenic stimulator cells, CTL were generated that specifically lysed mouse L cell (H-2^k) or human B cell targets expressing HLA-B27, and this lysis thus appeared largely unrestricted by H-2. These results indicate that transgenic mice express a functional HLA-B27 gene product on cell surfaces in the absence of the human β2-microglobulin gene. These transgenic mice promise to be a valuable resource in the investigation of the unique role of HLA-B27 in inflammatory human disease.

HLA-B27 is a serologically identified group of human class I MHC molecules, consisting of allelic 44,000 M_r glycoprotein H chains encoded by the *HLA-B* locus of the 6th chromosome, expressed on cell membranes in non-covalent association with a non-polymorphic 12,000 M_r L chain, β_2m[3], encoded on the 15th chromosome (1, 2). B27$^+$ individuals are at significantly greater risk than B27$^-$ individuals for developing any of several inflammatory disease states, including ankylosing spondylitis, reactive arthritis, or acute anterior uveitis (3, 4).

Although it remains formally possible that the actual disease susceptibility factor is a gene in tight linkage disequilibrium with HLA-B27, considerable evidence indirectly supports the hypothesis that the B27 gene product itself is involved (4–6). However, despite the fact that several micro-organisms have been identified as triggers of B27-associated disease in humans, the mechanism by which B27 molecules might participate in disease pathogenesis is completely unknown. Investigation of this mechanism would be facilitated by the availability of an experimental animal expressing the B27 gene.

Recently, Krimpenfort et al. (7) reported the introduction of two HLA-B27 genes into the germ lines of non-inbred mice. However, neither B27 gene product was expressed on cell surfaces in the host strains of mice. Expression was obtained in mice that were the progeny of crosses between transgenic B27 mice and transgenic mice bearing the human β_2m gene; only those progeny inheriting both genes expressed the B27 molecule on lymphoid cells, as detected by flow cytometry. The B27 gene product of these double transgenic mice was shown to function as a restricting element for anti-viral responses, as would be expected of a class I MHC product (8).

Although these findings represent a significant advance, it would nonetheless be more convenient to have a B27 transgenic animal model expressing a functional B27 gene product in conjunction with the endogenous β_2m. In addition, it would be advantageous for immunologic studies to have the B27 gene expressed on an inbred background. In this communication, we report the production of both inbred and non-inbred lines of transgenic mice, expressing physiologic levels of HLA-B27 in the absence of any other transgene. The B27 gene product was shown to be recognized as a class I MHC molecule by murine allogeneic CTL. These B27 transgenic lines will be valuable in the investigation of B27-associated disease and of class I MHC gene function.

MATERIALS AND METHODS

Mice. C57BL/6J (B6) and SJL/J mice were purchased from The Jackson Laboratories, Bar Harbor, ME, and (B6 × SJL) F_1 mice were bred in our animal facility. Outbred Swiss mice were purchased from Simonsen Labs, Gilroy, CA.

Genes. A genomic clone, pE.1-B27, containing a gene encoding an HLA-B27 molecule of the B27.1 subtype (2), was isolated from a λgt10 size-selected library from a B27-homozygous ankylosing spondylitis patient, as previously described (9). The 6.5-kb *Eco*RI insert was subcloned and propagated in pUC19.

Germ-line injection. The isolated insert of pE.1-B27 was microinjected into the male pronucleus of fertilized B6 or (B6 × SJL) F_2 eggs, which were transferred to pseudopregnant foster mothers, as previously described (10).

Identification of B27 transgenic mice. Genomic DNA was prepared from tail biopsies of weanling mice and hybridized on nitrocellulose filters with ^{32}P-labeled probes specific for the microinjected gene, as previously described (10). Two probes were used: a 0.6 kb *Eco*RI-*Xba*I fragment from the 5′ flanking region of the pE.1-B27

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[3] *Abbreviation* used in this paper: β_2m, β_2-microglobulin.

insert, and a 1.6 kb *XbaI-XbaI* fragment from the 3′ flanking region of an HLA-B7 gene, p12.1-B7 (9).

mAb and flow cytometry. Lymphoid cell surface expression of HLA-B27 was detected with the following mAb: ME1 (anti-HLA-B27) (11), and B9.12.1 and B.1.23.2 (both monomorphic anti-class I HLA) (12). H-2D^b was detected with the antibody 28-14-8S (13), and H-2^s molecules were detected with the antibody 3-83P (14). The mouse myeloma P1.17 was used as a non-specific control. B9.12.1 and B1.23.2 hybridoma lines were provided by Dr. F. Lemonnier, Inserm-CRNS, Marseille, France, and the antibodies were used as either hybridoma supernatant or affinity-purified antibody. The other hybridoma lines were purchased from the American Type Culture Collection, Bethesda, MD and used as affinity-purified antibody (ME1 and P1.17), hybridoma supernatant (28-14-8S), or clarified ascites (3-83P). After incubation of spleen or PBMC with mAb, the washed cells were incubated with FITC-labeled F(ab)′$_2$ goat anti-mouse Fc, or FITC-goat anti-mouse IgG (H and L chain) antibodies (Cappel, Malvern, PA). Cytofluorometry was carried out on a Becton Dickinson FACStar (Becton Dickinson, Mountain View, CA) as previously described (9). In all assays, the binding of the anti-HLA antibodies to nontransgenic (B6 × SJL) F$_1$ lymphoid cells and of the irrelevant antibody P1.17 to either non-transgenic or transgenic spleen cells gave superimposable negative patterns.

Cell lines. L cell transfections were carried out as previously described (9). The L cell line J26 (15), expressing the human β_2m gene, was provided by Dr. P. Kavathas, Yale University, New Haven, CT; the line 2.3.6, expressing high levels of HLA-B27, was produced by co-transfecting J26 cells with the plasmids pE.1-B27 and pSV2Neo, followed by selection with the antibiotic G418 (16). The human lymphoblastoid cell lines used were: MC (HLA-A1, A3, B8, B27), KASB (HLA-A1, A2, Bw57, Bw63), and Daudi (null HLA-A, B, C expression).

Allogeneic CTL responses. Primary in vitro MLC was carried out as previously described (17). Briefly, (B6 × SJL) F$_1$ responder spleen cells at 5 × 10^6 cells/ml were cultured for 5 days with an equal number of irradiated (3000 rad) transgenic B27 spleen cells. The secondary response was assayed following a similar 5-day MLC containing responder spleen cells from (B6 × SJL) F$_1$ mice primed i.p. 1 to 4 wk earlier with 3 × 10^7 B27 transgenic spleen cells. Both primary and secondary effector cells were tested in a 4-h ^{51}Cr release assay against transfected mouse L cell targets or human LCL targets. Data are presented as % cytotoxicity =

$$100 \times \frac{\text{isotope release by effector cells} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}$$

Spontaneous release for all targets ranged between 10 and 25% of maximal release. Each E/T combination was assayed in triplicate, and for each the SEM did not exceed 5%.

RESULTS

Integration and expression of HLA-B27 gene in (B6 × SJL) F$_2$ mice. From a total of 279 fertilized (B6 × SJL) F$_2$ ova, 64 live pups were obtained. Hybridization of genomic DNA from these mice demonstrated that eight had incorporated the HLA gene. Spleen cells from these eight were tested for cell surface expression of HLA-B27 by flow cytofluorometry. Four mice, numbers 11-5, 12-4, 18-3, and 21-5, showed substantial expression of HLA-B27, as detected with the mAb ME1. Spleen cells from two other mice showed slight reactivity with ME1, whereas spleen cells from the remaining two B27 mice showed no binding of ME1 above background (Table I).

The four mice with significant B27 expression were bred with non-transgenic (B6 × SJL) F$_1$ mice. Offspring were tested by DNA hybridization for germ-line inheritance of the B27 gene. Spleen cells from representative DNA$^+$ offspring were assayed by cytofluorometry for cell surface expression of B27 and the endogenous H-2^b and H-2^s molecules. As exemplified in Figure 1, all four founder animals produced progeny positive for the B27 gene at the level of genomic DNA. In each of the four cases, the expression of the B27 gene product showed similar cytofluorographic patterns in the founder mouse and its corresponding representative offspring.

In three of the lines, 12-4, 18-3, and 21-5, the spleen cell surface expression of HLA-B27 approximated that of the endogenous H-2 class I molecules. This can be seen in the cytofluorographs for the offspring mice in Figure 1, and is specifically demonstrated in Figure 2, in which background fluorescence has been subtracted out. Mouse 21-5-4, which was heterozygous for H-2^b and H-2^s, showed net mean channel fluorescence intensity for HLA-B27 that was virtually indistinguishable from that of either H-2 allotype.

Integration and expression of HLA-B27 gene in B6 mice. From a total of 332 micro-injected and transferred

TABLE I

Integration and expression of the HLA-B27 gene in transgenic mice

Recipient Strain	Ova Transferred	Offspring	
		B27 gene integrated/ total	B27 cell surface expression
(B6 × SJL) F$_2$	279	8/64	6
B6	332	11/28	4

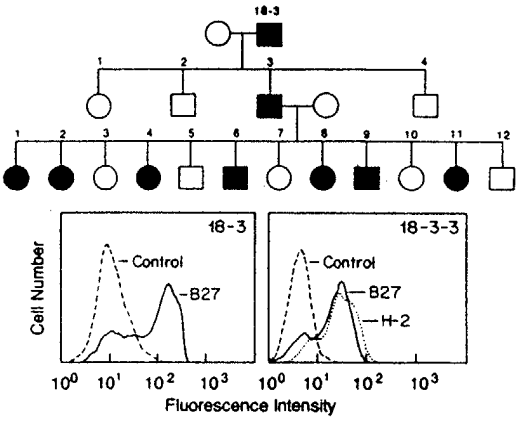

Figure 1. Inheritance of the HLA-B27 gene and cell surface expression of the gene product. The founder (B6 × SJL) F$_2$ mouse 18-3 was bred with a (B6 × SJL) F$_1$ non-transgenic mouse and the offspring tested for genomic integration of the B27 gene by DNA hybridization. One of 4 first generation offspring and 7 of 12 second generation offspring inherited the transgene, as indicated by the *closed symbols*. Spleen cells from the founder, 18-3, and offspring, 18-3-3, were tested in separate assays for cell surface expression of HLA-B27 with the mAb ME1, 20 μg/ml; in the case of the offspring, H-2D^b was assayed with the mAb 28-14-8S, at a 1/2 dilution of hybridoma supernatant. In the assay of 18-3 cells, the FITC-labeled second antibody was a goat anti mouse IgG H and L chain; background staining of splenic B cells by this antibody has been subtracted from the pattern shown. In the assay of 18-3-3 cells, the second antibody was specific for the IgG Fc fragment and showed no background staining.

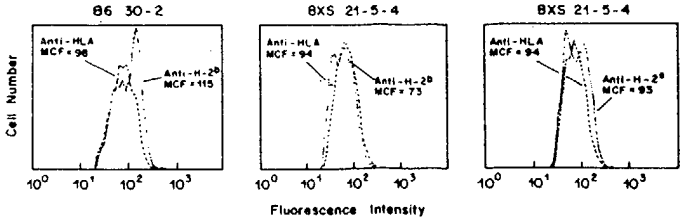

Figure 2. Comparison of cell surface expression of endogenous and transgenic class I MHC molecules. Spleen cells from the B6 mouse 30-2, the (B6 × SJL) F$_2$ mouse 21-5-4, and a control (B6 × SJL) F$_1$ mouse were assayed with the anti-HLA class I monomorphic antibody B.9.12.1 (1/2 hybridoma supernatant), anti-H-2D^b (28-14-8S, 1/2 hybridoma supernatant), or anti-H-2^s, (3-83P, 1/100 ascites). Background staining of the control spleen cells has been subtracted from the patterns, and mean channel fluorescence (*MCF*) of the positively staining population is indicated. Comparable expression of the B27 transgene product and of the endogenous class I H-2 molecules is demonstrated.

B6 ova, 28 live pups were obtained (Table I). Hybridization of genomic DNA from these mice demonstrated that 11 had incorporated the HLA gene. Spleen cells from nine of these were tested for cell surface expression of HLA-B27 by flow cytofluorometry. Four mice, numbers 26-3, 26-6, 30-2, and 30-4, showed substantial expression of HLA-B27, as detected with the monoclonal antibodies ME1, B9.12.1, and B.23.2 (Fig. 2). Three of these mice were bred with nontransgenic B6 mice. Offspring of all three showed germ-line inheritance and expression of the gene (data not shown).

Allogeneic recognition of HLA-B27 by cytolytic T cells. To assess recognition of the B27 transgene product as a class I MHC determinant by non-transgenic mouse spleen cells, primary and secondary in vitro MLC were established between non-transgenic responder (B6 × SJL) F_1 spleen cells and irradiated $B27^+$ transgenic stimulator cells. In this combination, the only stimulator Ag not shared by the responder is HLA-B27. After 5 days, the cultured cells were tested as cytolytic effectors against $B27^+$ and $B27^-$ mouse and human targets.

As shown in Figure 3, the B27-stimulated F_1 cells lysed $B27^+$ L cell transfectants to a significantly greater extent than $B27^-$ L cell transfectants. Because the responder cells were H-2^b × H-2^s and the L cell targets were H-2^k, it appears from this result that recognition of B27 in this system need not be H-2-restricted.

That this recognition was unrestricted by H-2 is further suggested by experiments such as those shown in Figure 4, in which F_1 effector cells lysed $B27^+$ human targets to a significantly greater extent than $B27^-$ targets. In other experiments, line 18-3 and line 12-4 cells were separately observed to generate anti-B27 CTL, and specific lysis of Daudi cells was <3% (data not shown).

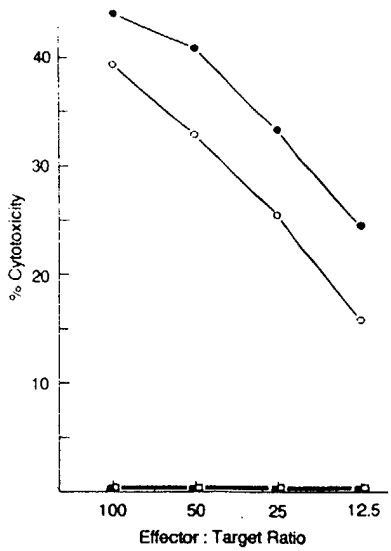

Figure 4. Cell-mediated cytotoxicity assay of primary and secondary (B6 × SJL) F_1 anti-B27 CTL against human targets. (B6 × SJL) F_1 spleen cells from naive donors (primary response) or donors primed 4 wk earlier with 3 × 10^7 $B27^+$ line 18-3 spleen cells (secondary response) were cultured in a 5-day MLC with irradiated $B27^+$ line 21-5 and 12-4 spleen cells, then assayed against human LCL targets: ○, ●, $B27^+$ targets of the primary and secondary responses, respectively; □, ■, $B27^-$ targets of the primary and secondary responses, respectively.

DISCUSSION

These experiments have demonstrated that introduction of an HLA-B27 gene into the germ line of both (B6 × SJL) hybrid mice and inbred B6 mice by micro-injection results in physiologic cell surface expression of the HLA-B27 gene product. Function of the B27 Ag as the stimulator of both primary and secondary allogeneic cytolytic T cell responses was also demonstrated; this result suggests that the human transgene product functions as a normal class I MHC molecule in the mouse.

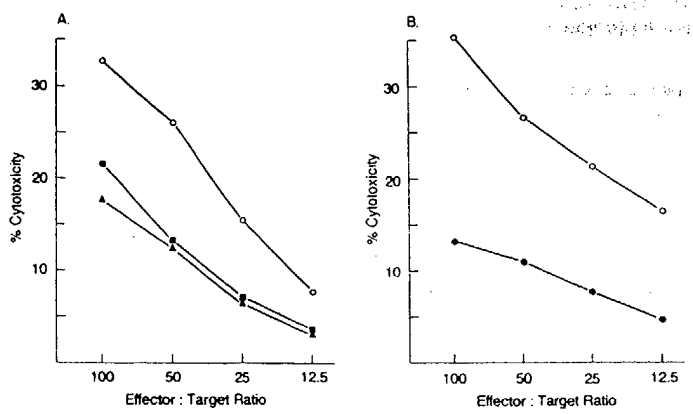

Figure 3. Cell-mediated cytotoxicity assay of primary and secondary (B6 × SJL) F_1 anti-B27 CTL against murine L cell targets. *A*, Non-transgenic F_1 cells were cultured 5 days in a primary MLC with irradiated $B27^+$ cells of the 18-3 line and assayed against transfected L cell targets. ○, 2.3.6 L cells transfected with HLA-B27, human β2m, and the selection marker thymidine kinase (tk); ▲, transfected with H-$2K^b$ (18) and tk; ■, transfected with tk alone. *B*, Non-transgenic F_1 mice were primed in vivo with 3 × 10^7 line 18-3 $B27^+$ spleen cells. After 4 wk, the primed F_1 spleen cells were cultured in a 5-day MLC with $B27^+$ irradiated stimulator cells from lines 21-5 and 12-4, then assayed against transfected L cell targets: ○, $B27^+$ 2.3.6 cells; ●, B27- J26 cells.

Inasmuch as intracellular association with β_2m is thought to be critical for the efficient transport of class I MHC H chain molecules to the cell membrane (19) as well as for recognition by the monoclonal antibody B.9.12.1 (12), the B27 transgene product is presumably transported to the cell surface in association with the endogenous murine β_2m. These results differ from those of Krimpenfort et al. (7), who reported a B27 transgene in non-inbred CBA × B6 mice that expressed a product detectable with the anti-HLA class I mAb W6/32 only in animals that also expressed a human β_2m transgene. It is possible that this difference in results can be explained by the fact that W6/32 binds much less well to the complex of HLA H chain and murine β_2m than to the complex of HLA H chain and human or bovine β_2m (20, 21). Alternatively, in the studies of Krimpenfort et al. (7) the presence of the human β_2m might have served to augment the expression of B27 in transgenic mice comparable to those of our mice that showed integration of the B27 DNA but were negative for B27 cell surface expression. This interpretation is supported by the report that the human β_2m gene augmented by 40-fold the cell surface expression of a transfected HLA-B7 gene in murine cells (22), and by our finding that mean channel fluorescence of spleen cells stained with B.9.12.1 or ME1 was ~10 fold greater in mice inheriting both the B27 and human β_2m genes than in mice inheriting only B27 (J. D. Taurog and R. E. Hammer, unpublished results).

Studies of the murine T cell response to HLA class I gene products expressed in human cells or transfected murine cells have suggested that murine anti-HLA CTL are largely restricted by H-2 (23, 24), and that the recognized entity may be HLA peptide fragments presented as

nominal Ag, rather than intact HLA class I cell surface molecules (25). Nonetheless, several aspects of our results suggest that the CTL response of (B6 × SJL) F_1 spleen cells to the B27 transgenic cells is largely a normal allogeneic response unrestricted by H-2. First, primary responses were observed. Second, these responses were increased by in vivo priming. Third, none of the target cells used shared H-2 Ag with either the stimulator or the responder cells. Fourth, since both mouse and human targets were lysed specifically, it is unlikely that co-recognition of a MHC Ag is involved. However, our studies have not excluded the possibility that an H-2-restricted response to HLA-B27 may also occur in MLC alongside the unrestricted response shown here.

Our results confirm and extend those recently reported by Chamberlain et al. (26), who observed expression of an HLA-B7 transgene in (B6 × SJL) F_2 mice in the absence of human β_2m and allogeneic recognition of the B7 product. Our B27 transgenic mice appear to have approximately threefold higher cell surface levels of transgene product, as assessed by comparing the relative cytofluorometric levels of transgene and endogenous H-2 gene expression in the two studies. In addition, whereas Chamberlain et al. (26) demonstrated generation of anti-HLA-B7 CTL in an MLC that was preceded by in vivo priming of the responder, we have demonstrated generation of anti-B27 CTL in a primary in vitro MLC. We have also observed physiologic expression of HLA-B27 in inbred C57BL/6 mice, which should prove to be an important resource for functional studies of the B27 gene product.

Although the mechanism of B27-associated disease in humans remains a mystery, several well-characterized gram-negative bacilli have been shown to trigger reactive arthritis, predominantly in $B27^+$ individuals (27, 28). The existence of inbred transgenic mice with physiologic expression of HLA-B27 provides a convenient experimental system in which to study in detail the interactions between HLA-B27 and known microbial triggers of reactive arthritis in humans.

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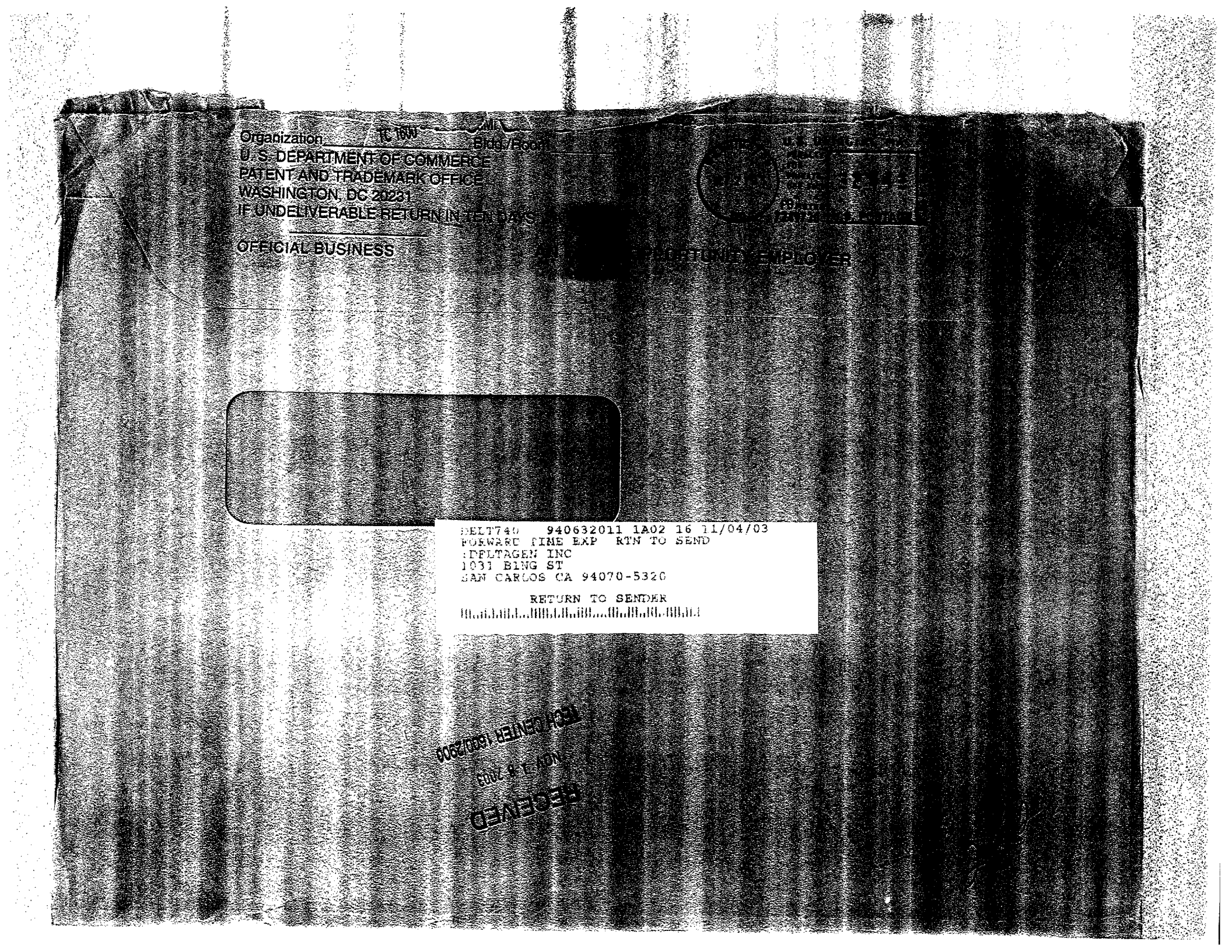
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